Universidade de Aveiro Departamento de Química
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Efeitos do envelhecimento no proteoma mitocondrial do músculo esquelético

Aging effects on the skeletal muscle mitochondrial proteome

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Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Métodos Biomoleculares, realizada sob a orientação científica do Doutor Francisco Manuel Lemos Amado, Professor Auxiliar do Departamento de Química da Universidade de Aveiro, e da Doutora Rita Maria Pinho Ferreira, Professora Coordenadora Convidada do Instituto Politécnico de Saúde - Norte

Apoio financeiro da FCT (PTDC/DES/70757/2006) no âmbito do III Quadro Comunitário de Apoio.

Dedico este trabalho à “Titi”.

o júri

presidente

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agradecimentos

Quero agradecer a todas as pessoas que de alguma forma contribuíram para realização deste trabalho.

Aos meus orientadores, Doutor Francisco Amado e Doutora Rita Ferreira, obrigado por me terem acolhido e por se dedicarem à difícil tarefa de pôr um "biólogo" a pensar nas "moléculas".

Ao Doutor Rui Vitorino, obrigado pelos valiosos ensinamentos de espectrometria de massa. Sei que não foi fácil, mas agora sinto que valeu o esforço de ambos para que eu aprendesse um pouco que fosse.

À Sofia, obrigado por estares disponível para aquelas pequenas explicações que fazem a diferença.

A todos os elementos do grupo de espectrometria de massa, um "muito obrigado" por me terem acolhido e pelos bons momentos que passámos.
Sei que a amizade não se vai esgotar por aqui e vamos continuar em contacto.

A todos os meus amigos que me apoiaram durante este período em que nem todos os momentos foram fáceis.

À minha família por terem permitido que chegasse a este ponto.

A todos, um sincero e sentido,

Obrigado

palavras-chave

Mitocôndria, proteómica, stress oxidativo, Maldi-Tof/Tof, cadeia transportadora de electrões

resumo

Este trabalho teve como objectivo determinar de que forma o envelhecimento afecta o proteoma mitocondrial do músculo-esquelético. Para tal, foram analisadas (i) alterações na composição (ii) variações da actividade dos complexos da cadeia respiratória, (iii) a oxidação de proteínas mitocondriais e (iv) os locais específicos de oxidação dos Complexo I da Cadeia de Transporte de Electrões (ETC). Simultaneamente, verificou-se de que forma o estilo de vida sedentário vs. não-sedentário pode condicionar cada um dos parâmetros observados. Para atingir estes objectivos ratinhos C57BL/6 machos foram sujeitos a um protocolo para simular o envelhecimento sedentário e não-sedentário. Os animais foram depois sacrificados e isoladas as mitocôndrias dos músculos dos membros traseiros. As proteínas mitocondriais foram depois separadas por 2D-PAGE e identificadas por espectrometria de massa. Após a comparação dos perfis de 2D-PAGE pode-se observar que a maioria das proteínas analisadas se encontra sobre-representadas no proteoma mitocondrial de ratinhos sedentários. Para avaliar se estas diferenças se correlacionavam com eventuais alterações na actividade dos complexos da cadeira respiratória, estes foram separados por BN-PAGE e corados com soluções específicas para determinar a sua actividade. Verificou-se uma diminuição na actividade dos complexos IV e V da cadeia respiratória que parece indicar que o aumento da quantidade de proteínas dos complexos da ETC no proteoma mitocondrial surge como um mecanismo de compensação para suprimir a diminuição da actividade da cadeia respiratória. Como parâmetro de oxidação proteica foi determinado o conteúdo em grupos carbonilos das amostras por *slot blot*. Verificou-se que há uma diminuição significativa do conteúdo em grupos carbonilos nas proteínas dos ratinhos que foram sujeitos a um estilo de vida não-sedentário. Para determinar os locais de oxidação do Complexo I da ETC, as sete subunidades deste complexo, identificadas previamente, foram sujeitas a espectrometria de massa *tandem*. Foram detectadas modificações em vários péptidos, sendo os resíduos de triptofano mais afectados pela oxidação. Contrariamente ao esperado, as subunidades membranares apresentaram-se mais modificadas que as subunidades mais próximas do centro catalítico. Os resultados permitem sugerir que as subunidades membranares são mais tolerantes aos efeitos da oxidação e que as subunidades do Complexo I podem ser substituídas individualmente quando são danificadas pelos efeitos da oxidação. Globalmente podemos concluir que a realização de actividade física moderada previne os efeitos do envelhecimento, nomeadamente a nível de danos oxidativos nas proteínas mitocondriais.

keywords Mitochondria, proteomics, oxidative stress, Maldi-Tof/Tof, electron transport chain

abstract This work aimed to assess how aging influences the skeletal muscle mitochondrial proteome. To achieve this, we have analyzed (i) alterations on its composition, (ii) variations on the activity of the respiratory chain complexes, (iii) the oxidation of mitochondrial proteins and (iv) the specific sites for oxidative modification on Complex I from the Electron Transport Chain (ETC). Simultaneously, we assessed how these parameters may be conditioned by a sedentary vs. a non-sedentary lifestyle. C57BL/6 male mice were subjected to a protocol to simulate sedentary and non-sedentary aging. The animals were then sacrificed and mitochondria from the hind limbs muscles were isolated. Mitochondrial proteins were resolved by 2D-PAGE and identified by mass spectrometry. Following a comparison of the 2DE profiles, we have observed that most of the analyzed proteins were up-regulated in sedentary mice mitochondrial proteome. To assess if these alterations were related to any functional variations, the respiratory chain complexes were resolved by BN-PAGE and differentially stained to determine the in-gel activity. We observed a reduction of the activity of Complexes IV and V in sedentary mice, which may suggest that the up-regulation of the respiratory chain complexes in sedentary mice may be a mechanism to overcome the loss of functionality. To assess the level of oxidative stress, we have determined the carbonyl content by slot blot and observed a significant decrease in the carbonyl content of non-sedentary mice. To locate the precise sites for oxidative damage in Complex I of the ETC, the seven subunits of this complex, previously isolated by 2D-PAGE, were subjected to tandem mass spectrometry. We have detected several modified peptides, with tryptophan being the most affected residue. Unexpectedly the membrane subunits presented more modification sites than the peripheral arm subunits. These results may suggest that membrane subunits are more tolerant to oxidative damage and Complex I subunits may be replaced by newly synthesized ones when oxidative damage becomes unbearable. In conclusion, we may assume that moderate physical activity attenuates the effects of aging, namely the oxidative damage of mitochondrial proteins.

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CHAPTER I – General Introduction

I. GENERAL INTRODUCTION

1. The aging process

"Aging is the progressive accumulation of changes with time that are associated with or responsible for the ever-increasing susceptibility to disease and death which accompanies advancing age" (Harman 1981). Aging refers to a multidimensional process of physical, psychological, and social change. During aging, organisms go through a series of alterations on every level, from molecules to the whole organism. The functional pathways involved in aging process may include responses to endogenous and exogenous changes, such like hormonal changes and damage accumulation; also, aging and longevity may be influenced by genes (Finch 1993; Finch and Tanzi 1997).

Biological, epidemiologic and demographic data have generated a number of theories that attempt to identify a cause or process to explain aging. These theories can be divided into four major categories, according to the biological level to which they apply, as evolutionary, molecular, cellular and systemic theories (Weinert and Timiras 2003). Some of the principal theories are listed in Table I.1 with a brief description.

Evolutionary theories argue that aging results from a decline in the force of natural selection. Longevity is a trait to be selected only if it is beneficial for fitness, as the natural selection acts primarily to maximize reproductive yield in an individual. The Mutation Accumulation Theory, for example, argues that some hereditary age-related diseases have not been selected against because the individuals bearing those genes reproduce long before the manifestation of the disease, thus being able to transmit their genetic information before natural selection could act (Medawar 1952).

Molecular theories argue that either altered gene expression on development genes or errors in the DNA replication, transcription or RNA translation accumulate and eventually lead to cell dead. While there is a basal rate or error in these events, if the cell looses the ability to repair errors, aberrant molecules accumulate and may induce cell death (Kanungo 1975; Dice 1993).

Cellular theories argue that is the cellular senescence the cause of aging. In this category falls: the Cellular Senescence-Telomere Theory, which

states that the increase in frequency of senescent cells, which may result from the telomere loss in chromosomes or cell stress, leads to aging of the organisms; the Hayflick Limit Theory established by Leonard Hayflick, which argues that cell are limited to a certain number of divisions and then die; and the Free Radical Theory, which will be described in detail later in this chapter (Dice 1993; Weinert and Timiras 2003).

The last category of theories is System theories, which argue that failures in physiological systems are the cause of aging. The Neuroendocrine Theory postulates that the neuroendocrine system eventually looses the ability to control the hormone production, and in fact the secretion of hormones and their effectiveness decline in advanced age. On the other hand, the Immunologic Theory argues that is the decline of the immune system that leads to aging and death (Weinert and Timiras 2003).

These categories, however, are often overlapping and the division is not always noticeable: age-related alterations on a molecular level, eventually lead to cellular alterations, which, in turn, lead to systemic failure with a strong reproductive and survival implications in evolution (Weinert and Timiras 2003). Despite all these theories, the search of a single cause for aging has been replaced by the view of aging as an extremely complex and multifactorial process (Kowald and Kirkwood 1996). A more comprehensive and realistic understanding of the aging process allow the division of aging theories in two major groups, genetic theories and stochastic theories (Weinert and Timiras 2003; Mota, Figueiredo et al. 2004). However, the division into two major categories is too general and less informative about the effects and their location.

Table I.1 – Classification and brief description of the main theories of aging (adapted from Weinert and Timiras 2003)

Biological level / Theory	Brief description
Evolutionary	
Mutation accumulation	Mutations that affect health at older ages are not selected against.
Disposable soma	Somatic cells are maintained only to ensure continued reproductive success; after reproduction, soma becomes disposable.
Antagonistic pleiotropy	Genes beneficial at younger age become deleterious at older ages.
Molecular	
Gene regulation	Aging is caused by changes in the expression of genes regulating both development and aging.
Codon restriction	Fidelity/accuracy of mRNA translation is impaired due to inability to decode codons in mRNA.
Error catastrophe	Decline in fidelity of gene expression with aging results in increased fraction of abnormal proteins.
Somatic mutation	Molecular damage accumulates, primarily to DNA/genetic material.
Dysdifferentiation	Gradual accumulation of random molecular damage impairs regulation of gene expression.
Cellular	
Cellular Senescence-Telomere Theory	Phenotypes of aging are caused by an increase in frequency of senescent cells. Senescence may result from telomere loss (replicative senescence) or cell stress (cellular senescence).
Free radical and reactive species	Oxidative metabolism produces highly reactive free radicals that subsequently damage lipids, protein and DNA.
Wear-and-tear	Accumulation of normal injury.
Apoptosis	Programmed cell death from genetic events or genome crisis.
System	
Neuroendocrine	Alterations in neuroendocrine control of homeostasis results in aging-related physiological changes.
Immunologic	Decline of immune function with aging results in decreased incidence of infectious diseases but increased incidence of autoimmunity.
Rate-of-living	Assumes a fixed amount of metabolic potential for every living organism (live fast, die young).

1.1. The Reactive Species Theory of Aging

One of the most popular explanations for how aging occurs at the biochemical level was firstly proposed by Denham Harman in what he called "the free radical theory of aging" in 1956 (Harman 1956). This theory, however should be called "the reactive species theory of aging" as, it does not only explain aging with the production of free radicals, but also with other

non-radical reactive species, namely reactive oxygen species (ROS) and reactive nitrogen species (RNS). Apart from the naming issues, it is still one of the best known theories of aging, although controversial. All organisms live in an environment that contains ROS; mitochondrial respiration, the major source of energy for all eukaryotes, generates ROS by leaking electrons from the electron transport chain (ETC). The production of the superoxide radical on the ETC is counterbalanced by the action of superoxide dismutase, an enzyme that is found in all aerobic organisms and scavenges superoxide anions exclusively (Finkel and Holbrook 2000). The free radical theory of aging supposes that free radical reactivity is inherent in biology and results in cumulative damage to all the cellular components and ultimately to cellular senescence. In fact, the level of oxidatively damaged DNA and proteins is elevated in aged organisms (Stadtman, Starke-Reed et al. 1992; Hamilton, Van Remmen et al. 2001; Wanagat, Cao et al. 2001).

Over the past decade, the Free Radical Theory of Aging has gained wide acceptance because numerous studies have shown a strong correlation between increasing age and the accumulation of oxidative damage to cellular macromolecules (Warner 1994; Sohal and Weindruch 1996; Hamilton, Van Remmen et al. 2001; Wanagat, Cao et al. 2001).

1.2. The mitochondrion and aging

Mitochondria are recognized as an essential member of the eukaryotic cell cytoplasm, responsible for generation of most of the "energy currency" for the cell, ATP (Yaffe 1999; Koopman, Verkaart et al. 2005; Frazier, Kiu et al. 2006; Logan 2006). Apart from this, mitochondria also harbour essential parts of the urea cycle and are crucial for the breakdown of fatty acids, the generation of heat, and the biosynthesis of heme, pyrimidines, amino acids, phospholipids, and nucleotides (Koopman, Verkaart et al. 2005; Frazier, Kiu et al. 2006).

One important aspect of mitochondria is its dynamic morphology (Knowles, Guenza et al. 2002). The earliest electron microscopic observations of mitochondria defined their basic morphology as double-membrane organelles, typically rod-shaped, and about 1 micrometer in length (Scheffler

2001). The inner membrane is folded into "cristae" that project into the region inside the inner membrane called the "matrix." The area in between the inner and outer membrane is termed the "intermembrane space" (Figure I.1).

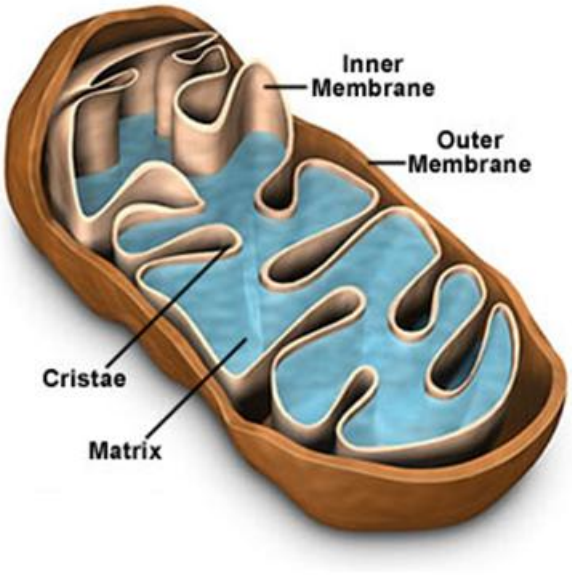


Figure I.1 - Schematic representation of a mitochondrion showing its internal configuration. (Adapted from http://www.cartage.org.lb)

Mitochondria display an amazing plasticity of form and distribution (Yaffe 1999; Collins, Berridge et al. 2002), which, along with its number, varies significantly between tissues and cell types and are further influenced by metabolic conditions and developmental stage (Rojo, Legros et al. 2002). The number of mitochondria in a cell is determined by the cell's specific function and energy needs. Cells such as muscle cells have many mitochondria while red blood cells have none. The distribution of mitochondria must strategically meet the cellular needs and signals from outside (Dimmer and Scorrano 2006). The mitochondrial plasticity may account for the discrepant observations of heterogeneity/homogeneity in mitochondrial form and function from different studies (Collins, Berridge et al. 2002). Furthermore, it emphasizes the conclusion that there is probably no single universal mitochondrial morphology and functional status and, even within an individual cell, we can have distinct populations with specific morphologies (Collins, Berridge et al. 2002).

The mammalian mitochondrion contains 4 to 5 copies of its own circular DNA molecule (mtDNA). This small molecule of 16 kb encodes for 37 genes, from which 13 are proteins, all belonging to the respiratory chain (Table I.2), 2 are rRNA (16S and 12S) and the remainder are mitochondrial tRNAs (Cogswell, Stevens et al. 1993; Taanman 1999; Wallace, Brown et al. 1999; Freyssenet, Irrcher et al. 2004; Hood, Irrcher et al. 2006; Cannino, Di Liegro et al. 2007). Although traditionally considered "naked" due to the lack of histones, a number of proteins interact with the mtDNA (Garesse and Vallejo 2001). The mtDNA has a significant contribute for mitochondrial function and integrity (Joseph, Rungi et al. 2004; Zeviani and Di Donato 2004). Defects in the synthesis of one of the 13 mtDNA- or nDNA-encoded respiratory subunits can lead to respiratory chain dysfunction and a wide range of pathogenic conditions, some of which affect skeletal muscle (Joseph, Rungi et al. 2004; Zeviani and Di Donato 2004).

Table I.1 – List of proteins encoded in the mitochondrial genome of *Mus musculus* obtained from the NCBI Genome database (http://www.ncbi.nlm.nih.gov/sites/entrez), accession number NC_005089, accessed on May 18th, 2008

Product Name	Length	Gi	GeneID	Locus
NADH dehydrogenase subunit 1	318	34538598	17716	ND1
NADH dehydrogenase subunit 2	345	34538599	17717	ND2
cytochrome c oxidase subunit I	514	34538600	17708	COX1
cytochrome c oxidase subunit II	227	34538601	17709	COX2
ATP synthase F_0 subunit 8	67	34538602	17706	ATP8
ATP synthase F_0 subunit 6	226	34538603	17705	ATP6
cytochrome c oxidase subunit III	261	34538604	17710	COX3
NADH dehydrogenase subunit 3	115	34538605	17718	ND3
NADH dehydrogenase subunit 4L	98	34538606	17720	ND4L
NADH dehydrogenase subunit 4	459	34538607	17719	ND4
NADH dehydrogenase subunit 5	607	34538608	17721	ND5
NADH dehydrogenase subunit 6	172	34538609	17722	ND6
cytochrome b	381	34538610	17711	CYTB

Even though mitochondria contain their own genome and protein synthesizing machinery they are only semi-autonomous (Freyssenet, Irrcher et al. 2004; Logan 2006). Indeed, proteome analysis points to the existence of about 800 (yeast) to 1500 (human) different proteins in mitochondria (Wiedemann, Frazier et al. 2004), although more conservative estimations point out to 1000 mitochondrial proteins (Scheffler 1999). This indicates that the mitochondrial genome specifies only a few mitochondrial proteins (approximately 1%) (Poyton and McEwen 1996; Wiedemann, Frazier et al. 2004). So, the majority of mitochondrial proteins are encoded in the nuclear genome, synthesized in the cytosol and imported into the mitochondria post-transcriptionally (Butow and Avadhani 2004; Freyssenet, Irrcher et al. 2004; Logan 2006). Nonetheless, some of these nuclear-encoded mitochondrial proteins are co-assembled by RNA molecules encoded by the mitochondrial genome (Poyton and McEwen 1996; Wiedemann, Frazier et al. 2004).

The delayed onset and progressive course of mitochondrial diseases suggest that mitochondrial function may decline with age. This hypothesis is supported by multiple reports of age-related declines in mitochondrial gene expression and oxidative capacity (Wallace 1999; Barazzoni, Zanetti et al. 2005). Being one of the most mitochondria enriched tissues, skeletal muscle is no exception. For example, structural and functional changes in muscle during aging occur in a wide range of species, from *C. elegans* to humans (Nair 2005). Impaired mitochondrial enzyme activity and reduced mitochondrial protein synthesis rate has been linked to these structural changes in aging skeletal muscle (Yarovaya, Kramarova et al. 2002; Barazzoni, Zanetti et al. 2005), but the molecular level at which these alterations occur is not completely elucidated. A reduced mtDNA copy number may contribute to reduced mRNA abundance, which results in reduced mitochondrial protein synthesis and enzyme activity (Yarovaya, Kramarova et al. 2002; Barazzoni, Zanetti et al. 2005; Nair 2005). Muscle fibre composition and oxidative metabolism have also been shown to influence skeletal muscle mitochondrial gene expression in adult animal models (Williams, Salmons et al. 1986). The

overall effect is a reduced capacity for oxidative phosphorylation. The decreased availability of ATP may contribute to an overall reduction in the remodelling process that involves the synthesis and breakdown of proteins, both of which are energy consuming reactions in muscle (Nair 2005). However, this analysis is even complicated by the fact that skeletal muscle groups are highly heterogeneous with respect to oxidative metabolism and function (Barazzoni, Short et al. 2000).

2. The Electron Transport Chain

The oxidative phosphorylation (OXOPHOS) is possible due to an electrochemical gradient between the matrix and the intermembrane space. Within the inner membrane, a series of membrane-associated electron carriers constitute the respiratory chain, and are organized through a growing redox potential (Murray, Taylor et al. 2003). The electron transport chain (ETC) is a group of four protein complexes (Complexes I-IV) and two mobile carriers, ubiquinone and cytochrome c. Together with a fifth complex, the ATP synthase (Complex V), they constitute the respiratory chain which is responsible for the production of energy to the cell in the form of ATP (Figure I.2).

This set of complexes is located in the inner membrane of mitochondria and is organized by their redox potential, which allows the flow of electrons from the primary donor to the final acceptor through a series of redox reactions (Liu, Fiskum et al. 2002).

2.1. Composition of the Electron Transport Chain

The four complexes that constitute the ETC are multisubunit protein complexes associated with prosthetic groups that allow the electrons to flow (Figure I.2).

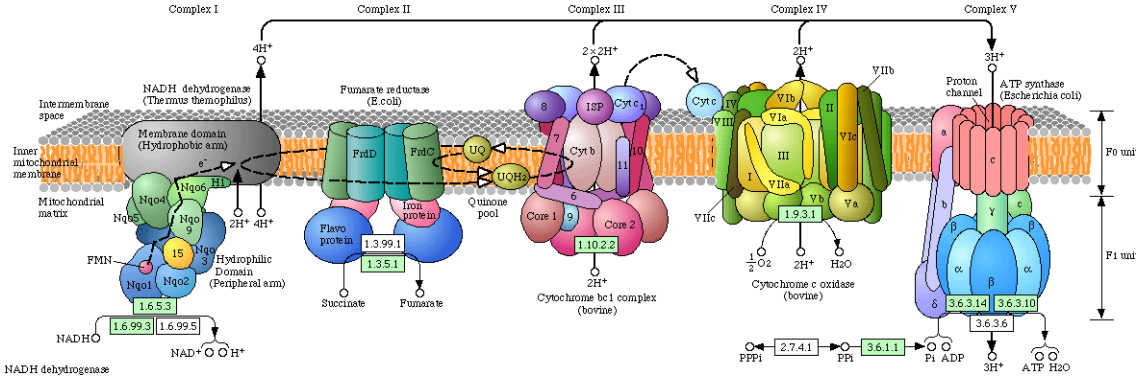


Figure I.2 - Schematic representation of the respiratory chain complexes published in Kyoto Encyclopaedia of Genes and Genomes (KEGG) Pathway website (http://www.genome.jp/dbget-bin/get_pathway?org_name=mmu&mapno=00190). Due to the lack of complete tridimensional structures for each complex of Mus musculus, representations are based on the respective complexes of other organisms: Complex I is based on *Termus temophilus*, Complex II and V on *Escherichia coli* and Complexes III and IV on bovine.

Complex I (C-I) is a NADH:ubiquinone oxidoreductase that catalyses the reduction of NADH to NAD^+, transferring two electrons to ubiquinone. It has an L-shaped form, with the long arm as a hydrophobic integral membrane protein and the short arm protruding to the matrix, which contains the FMN-containing flavoprotein and the iron-sulphur clusters (Navarro and Boveris 2007).

Complex II (C-II) is a succinate:ubiquinone oxidoreductase that reduces succinate to fumarate, transferring two electrons to ubiquinone. This complex is simultaneously part of the ETC and TCA cycle. It is an oligommeric complex and each monomer is composed of 4 subunits namely, a flavoprotein, with a FAD molecule covalently bound, an iron-sulphur protein and two hydrophobic smaller peptides that serve as membrane anchors. Complex II is the only complex in the respiratory chain that does not span the inner membrane and whose genes are only nuclear encoded (Cecchini 2003).

The mobile carrier between C-I/II and C-III is a lipid soluble benzoquinone with a long isoprenoid tail that may exist in three oxidation states: ubiquinone – fully oxidized, semiquinone – semi-oxidized, or ubiquinol – fully reduced. The most common form in mammals has 10 isoprene units and is referred as Coenzyme Q_{10} (Navarro and Boveris 2007).

Complex III (C-III) is an ubiquinol:cytochrome c oxidoreductase, hence it is responsible for the transfer of electrons to cytochrome c from ubiquinol. This complex has 9-10 polypeptides, three of which are associated with the redox centres b_{562}, b_{566} and c_1 hemes and an iron-sulphur cluster (Hatefi 1985). The membrane spanning region of each C-III monomer consists of 13 transmembrane helices, eight of which belong to cytochrome b (Xia, Yu et al. 1997).

Cytochrome c (cyt c) is a small peptide bound to a heme c group that is loosely associated to the mitochondrial inner membrane, facing the intermembrane space. It is reduced by the electrons transferred from C-III, transporting them to C-IV, where it is oxidized (Navarro and Boveris 2007).

Complex IV (C-IV) or cyt *c* oxidase (COX) is the final catalyst of the ETC. C-IV reduces O_2 to H_2O with four electrons from the reduced cytochrome c, consuming four protons from the matrix (Navarro and Boveris 2007). The redox centres of C-IV are two heme *a* centres, heme *a* and heme a_3, located in two different environments, each one associated with a copper atom, respectively, Cu_a and Cu_{a3} (Hatefi 1985).

2.2. The electron flow along the chain

The primary electron donors can be either NADH or succinate, creating two different pathways. When using NADH as the electron donor, the chain starts in Complex I; there, a pair of electrons is transferred to the flavine mononucleotide (FMN), leaving the NADH reduced to NAD^+. The electrons then flow through a series of eight iron-sulphur clusters reaching ubiquinone that is reduced to ubiquinol and heads out to Complex III. Contrastingly, when the electron donor is succinate, the chain starts in Complex II. Through this pathway, the succinate produced in Krebs cycle is reduced to fumarate by transferring two electrons to a flavine-adenine dinucleotide (FAD) in Complex II. Similarly to the NADH-pathway, the electrons flow from the FAD to ubiquinone through three iron-sulphur clusters. From this point on, both pathways follow the same steps. Complex III is responsible for the transfer of a pair of electrons from ubiquinol to two molecules cytochrome c. This latter will, in turn, transfer the electrons to Complex IV where the final reduction of

the chain occurs, and one molecule of O_2 is reduced to two molecules of water (Hatefi 1985).

One important feature that results from the flow of electrons along the chain is the transfer of protons from the matrix to the intracellular space. This transfer occurs against the electrochemical potential and is only possible because it is coupled with the redox reactions of the electron transport; otherwise it would require ATP to be achieved. The accumulation of protons on the intermembrane space creates an electrochemical potential against the matrix. This electrochemical potential is extremely important as protons return to the matrix through a specialized complex – the ATP synthase – which uses the energy released by the proton flow, back to the matrix, to phosphorylate ADP and thus produce ATP (Hatefi 1985).

During the flow of electrons, one may consider an internal cycle that occurs in Complex III, which is called the ubiquinone cycle and was proposed by Mitchell (1976). Complex III has two binding sites for ubiquinone and during the cycle both binding sites are occupied. When ubiquinol is bound to the first site, and an oxidized ubiquinone is bound to the second site, the protons of ubiquinol are stripped off and expelled at the intermembrane space. One of its electrons is passed on first to a heme within complex III and then to cytochrome c; the other electron is passed on to the second molecule of ubiquinone, which is reduced to semiquinone. The ubiquinol molecule is now oxidized to ubiquinone and is replaced by a new molecule of ubiquinol that has been reduced in the preceding steps of the ETC. The protons and electrons of the new ubiquinol molecule are abstracted and split as for the first molecule and we now have a fully reduced ubiquinol in the second site. The molecules then switch places and thereby complete the cycle (Figure I.3) (Mitchell 1976). However, the electron flow through the ETC is not error proof.

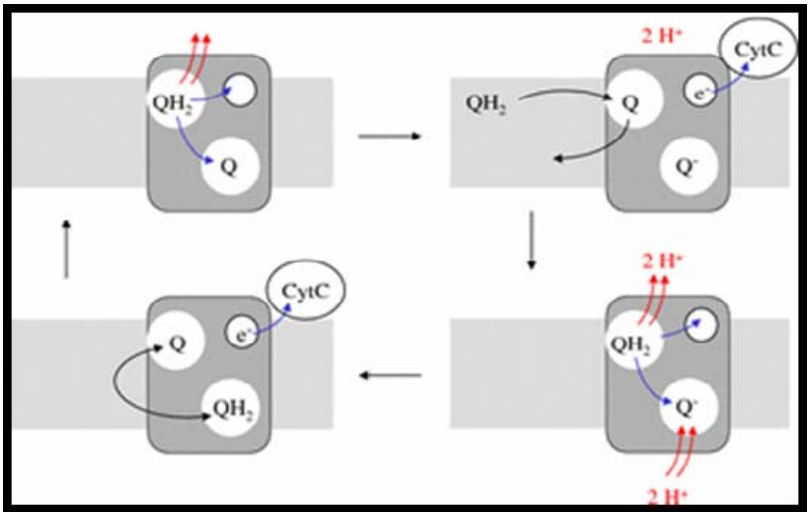


Figure I.3 – Schematic representation of the ubiquinone cycle. Complex III has two binding sites for ubiquinone. Oxidation of one molecule of ubiquinone partially reduces the second one; oxidation of a new molecule of ubiquinone completes reduction of the second one and causes uptake of two protons from the cytosol. Protons released by oxidation are at all times released at the cytosolic side. (obtained from http://watcut.uwaterloo.ca/webnotes/Metabolism/page-5.2.4.html)

2.3. Production of reactive species

During the electron flow along the chain, it may take place some electron leakage. By leaking from the normal flow, the electrons immediately react with O_2 in the matrix to produce superoxide radical ($O_2^{\bullet -}$). Current estimations state that up to 5% of the oxygen consumed with ATP production on the respiratory chain, can be converted into $O_2^{\bullet -}$ during a normal physiological state (Joenje 1989; Leeuwenburgh and Heinecke 2001; Wanagat, Cao et al. 2001). The biological toxicity of superoxide is, namely, due to its capacity to inactivate iron-sulphur cluster containing enzymes (which are critical in a wide variety of metabolic pathways), thereby liberating free iron in the cell, which can undergo Fenton chemistry and generate the highly reactive hydroxyl radical ($^{\bullet}OH$). In its hydroperoxyl radical form ($^{\bullet}OOH$), superoxide can also initiate lipid peroxidation of polyunsaturated fatty acids. It also reacts with carbonyl compounds and halogenated carbons to create toxic peroxy ($ROO^{\bullet}$) or alkoxy ($RO^{\bullet}$) radicals. Moreover, superoxide can also react with nitric oxide ($^{\bullet}NO$) to form peroxynitrite ($ONOO^{-}$). All these radicals and reactive species can induce damage in surrounding molecules such as proteins, nucleic acids, and lipids in a cascade, leading to the production of

more reactive species (Joenje 1989; Leeuwenburgh and Heinecke 2001; Wanagat, Cao et al. 2001; Stadtman and Levine 2003; Brookes 2005; Stadtman 2006).

2.4. Neutralization of reactive species - The antioxidant system

To counterpart the production of the reactive species, the cell developed mechanisms to neutralize them, such as enzymes or other molecules. The major mitochondrial enzymatic antioxidants are manganese superoxide dismutase, peroxiredoxin III, thioredoxin and thioredoxin reductase (Rabilloud, Heller et al. 2001).

Nearly all organisms living in the presence of oxygen contain isoforms of the superoxide scavenging enzyme, superoxide dismutase (SOD). SOD is a protein with metallic cofactors; eukaryotic cells have at least two forms of this enzyme, one is cofactored with copper and zinc and is located on cytosol, the other is cofactored with manganese and is located on mitochondria. Mn-SOD is an extremely efficient enzyme; it catalyzes the neutralization of superoxide nearly as fast as the two can diffuse together spontaneously in solution. The neutralization of superoxide is achieved by converting it into hydrogen peroxide (Warner 1994; Yamakura, Taka et al. 1998; Leeuwenburgh and Heinecke 2001).

Two systems have been proposed to neutralize hydrogen peroxide. The glutathione system includes glutathione, glutathione reductase, glutathione peroxidases and glutathione S-transferases, and was already shown to be present in mitochondria (Arai, Imai et al. 1996). Glutathione is a tripeptide (γ-glutamyl-Cys-Gly) that reduces any disulfide bonds formed within cytoplasmic proteins to cysteines by acting as an electron donor. Glutathione reductase recycles oxidized glutathione to its reduced form, using the electrons from NADPH. Glutathione peroxidase contains four selenium-cofactors that catalyze the breakdown of hydrogen peroxide and organic hydroperoxides. In addition, the glutathione S-transferases are another class of glutathione-dependent antioxidant enzymes that show high activity with lipid peroxides. These enzymes are at particularly high levels in the liver and also serve in detoxification metabolism. However, its presence in

mitochondria seems to be limited to the intermembrane space and in small amounts (Arai, Imai et al. 1999). The other proposed system includes peroxiredoxins and its regeneration system composed by thioredoxin and thioredoxin reductase (Rabilloud, Heller et al. 2001). Peroxiredoxins are peroxidases that catalyze the reduction of hydrogen peroxide, organic hydroperoxides, as well as peroxynitrite. They are divided into three classes: typical 2-cysteine peroxiredoxins; atypical 2-cysteine peroxiredoxins; and 1-cysteine peroxiredoxins. These enzymes share the same basic catalytic mechanism, in which a redox-active cysteine (the peroxidatic cysteine) in the active site is oxidized to a sulfenic acid by the peroxide substrate. The thioredoxin system contains the 12-kDa protein thioredoxin and the enzyme thioredoxin reductase. The active site of thioredoxin consists of two neighboring cysteines, as part of a highly-conserved CxxC motif, which can cycle between an active dithiol form (reduced) and an oxidized disulfide form. In its active state, thioredoxin acts as an efficient reducing agent, scavenging reactive oxygen species and maintaining other proteins in their reduced state. After being oxidized, the active thioredoxin is regenerated by the action of thioredoxin reductase (Lee, Yu et al. 1999; Lee, Kim et al. 2003).

Catalase is commonly localized in peroxisomes of most eukaryotic cells; however it was already demonstrated to be present in mitochondria (Nohl and Hegner 1978; Nohl and Jordan 1980; Radi, Turrens et al. 1991). This enzyme efficiently catalyses the conversion of hydrogen peroxide to water and oxygen, using either an iron or manganese cofactor. Lack of catalase in mitochondria of most cells can lead to the inefficient degradation of H_2O_2 in mitochondria, especially under conditions of oxidative stress where the mitochondria generate more H_2O_2 than under normal conditions (Bai and Cederbaum 2001).

Another major group involved in the antioxidant response includes representatives of heat shock proteins (HSP), namely HSP27, HSP60 and the family of HSP70. The expression of these proteins is induced by heat shock and several other stressors, including oxidative stress (Fehrenbach and Niess 1999; Liu, Gampert et al. 2006). The family of HSP70 is represented by four members, the constitively expressed isoform heat shock cognate (HSC70), the inducible isoform HSP70 (HSP72) and two glucose regulated isoforms GRP75

and GRP78. Up-regulation of HSP72 has been shown to provide protection from ischemia–reperfusion-induced lipid peroxidation in rat myocardium (Demirel, Powers et al. 1998). Furthermore, overexpression of mouse HSP25 and human HSP27 exerts augmenting effects on cellular levels of GSH by increasing the activity of glutathione reductase and glucose-6-phosphatase dehydrogenase (Preville, Salvemini et al. 1999).

An imbalance between oxidants and antioxidants in favour of the oxidants, potentially leading to damage, is termed 'oxidative stress' (Sies 1991; Sies 1997). As stated before, the superoxide radical is the main source of reactive species because is starts a chain of events that lead to the production of more reactive species. Its main production site is the ETC, whenever electron leakage occurs. Two sites within the ETC have been identified as being the major sites for the electron leakage to take place, and hence be the sites for superoxide radical formation. These critical sites are the area near the redox centres of Complex I and the ubiquinone binding sites in Complex III. Thus, the reduction of NADH and the ubiquinone cycle seem to be critical events in the radical formation (Liu, Fiskum et al. 2002; Choksi, Nuss et al. 2007; Choksi and Papaconstantinou 2008).

3. Protein oxidation in mitochondria

The oxidative modification of proteins is a natural consequence of aerobic life and have also been discussed as regulatory mechanisms in oxidative stress signalling (Stadtman and Levine 2003; Viappiani and Schulz 2006; Chung, Miranda et al. 2008). Protein oxidation is also recognized to play a crucial role in the etiology and pathophysiology of some diseases (Stadtman and Levine 2003; Viappiani and Schulz 2006; Chung, Miranda et al. 2008).

The oxidative modifications of a protein can range from the simple oxidation of cysteine residues to changes caused by higher levels of oxidative stress, resulting in covalent crosslinking with other proteins, the formation of noncovalent aggregates or even formation of protein adducts with other lipid, carbohydrate, or nucleic acid radicals (Viappiani and Schulz 2006; Choksi and Papaconstantinou 2008). Studies on oxidatively modified proteins have

revealed an age-related increase in the level of protein carbonylation (Levine 2002; Chung, Miranda et al. 2008), oxidized methionine (Wells-Knecht, Lyons et al. 1997), cross-linked (Squier and Bigelow 2000) and glycated proteins (Baynes 2001) as well as catalytically less active enzymes (Rothstein 1985) that are more susceptible to protein degradation (Stadtman 2001).

One of the best-known markers of age-related protein oxidation is the carbonyl group content, which has been observed to increase with age (Levine 2002). The main carbonyl products of metal-catalyzed oxidation of proteins *in vitro* have been shown to be glutamic and aminoadipic semialdehydes (Requena, Levine et al. 2003; Pamplona, Dalfo et al. 2005; Soskic, Groebe et al. 2008). Lysine, arginine, proline, and threonine residues of proteins are particularly sensitive to metal-catalyzed oxidation, leading in each case to the formation of carbonyl derivatives. Peptide carbonyl derivatives are also obtained as fragmentation products of peptide bond cleavage reactions or can be formed by the interaction of protein amino acid side chains (cysteine sulfhydryl groups, histidine imidazole groups, and lysine amino groups) with lipid peroxidation products, including 4-hydroxy-2-nonenal, acrolein, and malondialdehyde. Glycation/glycoxidation reactions can also lead directly to carbonyl adducts and indirectly to the formation of *N*-carboxymethyl-lysine derivatives that, because of their strong chelating ability, are able to promote the generation of carbonyl groups by metal-catalyzed reactions (Stadtman and Levine 2003; Stadtman 2006).

In a recent study by Chung et al. (2008), several carbonyl-modified proteins in interfibrillar rat heart mitochondria were identified as putative targets of oxidation modifications. These authors observed age-associated changes of carbonyl levels for aconitate hydratase and the α- and β-polypeptide chains of the F_1-ATP synthase. However, these studies did not identify the precise location in the protein structure where these oxidative modifications occur.

Much of the knowledge about free radical–mediated post-translationally modified products in mitochondria is derived from *in vitro* studies (Stadtman and Levine 2003; Taylor, Fahy et al. 2003). Tyrosine residues may be oxidized by hypochlorite, peroxynitrite or by radicals formed in transition metal

ion-catalyzed Fenton and Haber-Weiss reactions (e.g. hydrogen peroxide/Fe^{2+}). The resulting tyrosyl radicals may subsequently form intra- or intermolecular Tyr–Tyr bonds (Kowald and Kirkwood 1996; Balasubramanian and Kanwar 2002; Soskic, Groebe et al. 2008). The exposure of mitochondria or proteins to simulated oxidative stress *in vitro* such as peroxynitrite-mediated nitration suggested that tyrosine from proteins like manganese superoxide dismutase is a susceptible residue (Yamakura, Taka et al. 1998; Taylor, Fahy et al. 2003).

Tryptophan is another residue commonly affected by oxidative modification. The double oxidation of tryptophan residues generates N-formylkynurenine, this can be generated enzymatically and non-enzymatically (Yamakura, Taka et al. 1998; Taylor, Fahy et al. 2003; Korlimbinis and Truscott 2006; Soskic, Groebe et al. 2008). In one of the few *in vivo* studies about protein oxidation, Taylor et al. (2003) have examined the occurrence of *N*-formylkynurenine throughout the mitochondrial proteome of normal human heart tissue and found evidence of selective oxidation in a subset of proteins associated predominantly with redox metabolism, including ETC proteins such as six Complex I subunits (75kDa, 30kDa, 23kDa, 20kDa, 18kDa and 13 kDa-B), two Complex III subunits (subunit IV and core protein 2), Complex IV subunit IV and ATP synthase γ and δ chain subunits.

Generally, these oxidative modifications to proteins can result either in reduction of normal function or in the gain of toxic function related with aging and age-associated diseases (Stadtman and Levine 2003). The unwanted accumulation of oxidized proteins in such conditions could reflect random DNA damage to one or more of the multitude of genes that are implicated in the synthesis of proteins that govern the generation of ROS, the antioxidant defence systems, and the proteolytic activities that degrade oxidized proteins, therefore being important reliable molecular markers of aging (Chao, Ma et al. 1997; Stadtman and Levine 2003).

While important to cellular function, post-translational modifications, particularly oxidation, are often difficult to detect using analytical techniques such as gel electrophoresis and mass spectrometry. These difficulties arise

from a number of reasons including the lower relative abundance of modified protein when compared to the unmodified protein (Distler, Kerner et al. 2007).

4. Skeletal muscle aging is influenced by physical activity

Living an active lifestyle and at least maintaining mobility is essential for quality of life during old age (Ji 2002; Paterson, Jones et al. 2007). Skeletal muscle, a primary organ for locomotion, seems to be more prone to the deleterious effect of aging due to its post-mitotic nature (Sastre, Pallardo et al. 2003; Figueiredo, Ferreira et al. 2008). This age-associated deterioration, common in elderly humans and animals, is often referred to as sarcopenia. The term sarcopenia is defined as a loss of skeletal muscle mass and functionality that occurs with advancing age (Morley, Baumgartner et al. 2001; Carmeli, Coleman et al. 2002; Chabi, Ljubicic et al. 2008). This condition results in muscle weakness predicting several adverse outcomes, including disability, institutionalization and mortality (Carmeli, Reznick et al. 2000; Carmeli, Coleman et al. 2002; Short, Bigelow et al. 2005; Marzetti and Leeuwenburgh 2006). According to Carmeli and Reznick (1994), a variety of intrinsic and extrinsic factors appears to be involved in the aging skeletal muscle. Changes in intrinsic factors associated with aging include hormones, growth factors and energetic metabolism, whereas extrinsic factors include diet, exercise, injuries, and sedentary lifestyles. At a molecular level, it has been described altered gene expression, loss of cell division potential, increased protein degradation, tissue disorganization and increased vulnerability to stress (Yarovaya, Kramarova et al. 2002; Barazzoni, Zanetti et al. 2005; Nair 2005; Figueiredo, Ferreira et al. 2008; Figueiredo, Mota et al. 2008).

Mitochondria are intimately linked to the proper function of skeletal muscle, as these organelles constitute the main energy supply in contracting muscle (Chabi, Ljubicic et al. 2008). Dysfunctional mitochondria will be unable to meet cellular ATP demands compromising the cellular adaptability to physiological stress imposed to skeletal muscle across the entire lifespan, contributing to age-related muscle dysfunction and reduced aerobic capacity

(Short, Bigelow et al. 2005; Figueiredo, Ferreira et al. 2008). Several attempts to revert this situation have been made. In this sense, several studies discussed the effect of lifestyle on sarcopenia and on skeletal muscle mitochondrial plasticity. One of the classic responses to exercise is an increase in the oxidative capacity of skeletal muscle (Menshikova, Ritov et al. 2006). However, the merits of exercise for the well-being and maintenance of muscle tissue in old age are not always clear and depend on energy requirements, oxygen consumption and mechanical load predisposed by the type of exercise (Carmeli, Coleman et al. 2002). Moderate exercise was shown to increase muscular mass and strength, which counterparts for the loss of muscle mass associated with aging (Leeuwenburgh, Fiebig et al. 1994).

Moderate voluntary exercise has already been associated with an improvement in general health, increasing lifespan and improving neuromuscular coordination (Holloszy, Smith et al. 1985; Navarro, Gomez et al. 2004). This type of exercise also increases the activity of respiratory chain. In fact, it has been demonstrated that Complex IV activity suffered an increase in several tissues of mice subject to 24 weeks of chronic exercise, while the other Complexes showed no significant differences (Navarro, Gomez et al. 2004). On the other hand, Complexes I and IV showed a decrease in activity associated with aging (Navarro, Gomez et al. 2005), which was stopped by regular moderate exercise (Navarro, Gomez et al. 2004). Indeed, chronic moderate exercise was already shown to decrease the mitochondrial content in protein carbonyls and oxidation products (Navarro, Gomez et al. 2004).

5. Methodological approaches on the study of mitochondrial aging

The pivotal role of mitochondria in the aging process is still controversial. Although cytochrome c oxidase deficient fibres are a real finding in skeletal muscle, the contribution of mitochondrial DNA mutations to human aging is not as clear (Brierley, Johnson et al. 1997). Indeed, other studies have failed to prove changes associated with age in mitochondria (Manzelmann and Harmon 1987; Bodenteich, Mitchell et al. 1991).

These controversies can be explained by that fact that most of these studies come from genetic analysis, namely polymerase chain reaction (PCR) analysis on mtDNA and nuclear DNA encoding for mitochondrial proteins (Cortopassi and Arnheim 1992). The genomic analysis is not sufficient to fully elucidate the mechanisms of aging. Expression levels of a protein depend not only on transcription rates of the gene, but also on additional control mechanisms, such as transcript stability, translational regulation and protein degradation. Moreover, both the activity and the function of proteins can be altered, mainly through post-translational modification (glycosylation, phosphorylation) or proteolytic cleavage (Boguski and Schuler 1995; Amson, Nemani et al. 1996; Harry, Wilkins et al. 2000). All these points could contribute to the presence of largely controversial observations using genetic analysis (Rustin, von Kleist-Retzow et al. 2000; Storm, Rath et al. 2002). Thus it is imperative to analyze other levels to disclose the aging mechanisms. Proteome analysis come as an obvious approach, as most of the alterations occurring during aging are due to post-translational events as previously stated (Stadtman and Levine 2003).

High throughput two-dimensional protein electrophoresis coupled with peptide mass fingerprinting analysis by mass spectrometry (MS) have become the most powerful techniques for modern proteome analysis (Gras, Muller et al. 1999). The mitochondrial proteome has been studied in tissues like the human placent cells or heart muscle (Rabilloud, Kieffer et al. 1998; Taylor, Warnock et al. 2002) and cell lines (Fountoulakis and Schlaeger 2003). As stated above, the number of mitochondrial proteins is estimated to be around 1000-1500. One of the largest proteomic studies of purified mitochondria was presented by Sickmann, Reinders et al. (2003) on yeast mitochondria, leading to the identification of 750 mitochondrial or mitochondria-associated proteins with a coverage of up to 90% of predicted yeast mitochondrial proteome. More recently, Zhang, Li et al. (2008) identified a total of 940 distinct proteins from murine cardiac mitochondria, among which, 480 proteins were not previously identified by major proteomic profiling studies.

The sensitivity and effectiveness of proteomic analysis has only recently risen through a whole new repertoire of high-throughput technical

developments. Especially, large-gel 2D electrophoresis analysis has now reached a technical state that offers the possibility to reveal the majority of the cellular proteins with reproducible results (Klose, Nock et al. 2002). Blue-native electrophoresis has greatly contributed to the mitochondrial protein complex investigations (Schagger and von Jagow 1991), while Western immunoblotting remains to be an effective proteomic strategy. New protein analytical methods such as mass spectrometry compatible for macromolecules, computational tools, and comprehensive databases for characterization of molecular structures of proteins led to large-scale strategies in protein identification.

Maldi-TOF/TOF made large-scale proteomics possible. By coupling a Maldi source with a TOF analyzer it is possible to analyse large biomolecules quickly and efficiently without ripping them apart (Constans 2005). To study proteins we need to isolate them and then digest with an enzyme, usually trypsin, that cleaves the protein in specific residues (after any Arg or Lys for trypsine). The number and size of peptides resulting from tryptic digestion of a protein is called the peptide mass fingerprint (PMF) and is specific for each protein, helping us to identify it. The number of peptides observed in a PMF and the accuracy to which they are measured determine the confidence of the protein identification (Westermeier and Naven 2002).

There are databases with the fragment pattern for almost every known protein. Mascot is one of the best known search engines that use the data produced by mass spectrometers to identify proteins. It incorporates code from Mowse algorithm, developed by Darryl Pappin (1993) and David Perkins (1999). It can quickly return the most probable protein to be in a given sample with a given set of mass peaks on a mass spectrum. Additionally, it allows us to introduce variable modifications in the search, such as oxidations, which produce mass shifts and alter the fragmentation pattern of a given protein.

Thus, a proteomic approach seems an attractive strategy of studying complex biology problems such as aging, in order to gain additional knowledge of protein localization, protein interaction and their influence on protein structure and function.

6. Aims of this thesis

Given the importance of mitochondria in the aging process we aim to characterize the alterations induced by aging in mice skeletal muscle mitochondrial proteome.

To achieve this we have:

- Identified how aging affects the constitution of the mitochondrial proteome;
- Identified which are the proteins most susceptible to oxidative damage;
- Determined how the activity of the respiratory chain complexes is affected by aging;
- Identified the location of oxidative modifications on Complex I subunits from the ETC, and relate these with the changes in the activity.

Ultimately, by comparing the group of sedentary vs. non-sedentary mice, we have assessed the implications of a sedentary lifestyle on mitochondrial functionality and protein oxidative damage.

CHAPTER II – Sedentary lifestyle modulates the aging effect on mice skeletal muscle mitochondrial proteome

II. SEDENTARY LIFESTYLE MODULATES THE AGING EFFECT ON MICE SKELETAL MUSCLE MITOCHONDRIAL PROTEOME

1. Introduction

Skeletal muscle, a primary organ for locomotion, was shown to undergo age-associated deterioration in structure and function (Ji 2002; Johnston, De Lisio et al. 2008). Several strategies have been employed to revert this scenario enhancing endogenous antioxidant levels through dietary restriction, dietary antioxidant supplementation, and use of pharmaceutical antioxidant mimetics. However, none have been shown to successfully boost antioxidant defense in skeletal muscle (Ji 2002). Nonexhaustive exercise appear as a potent and effective countermeasure for skeletal muscle aging (Navarro and Boveris 2007; Paterson, Jones et al. 2007; Gomez-Cabrera, Domenech et al. 2008; Johnston, De Lisio et al. 2008). Indeed, moderate voluntary exercise was already shown to produce an increase in the lifespan of rodents (Holloszy, Smith et al. 1985), as well as improve neuromuscular coordination and exploratory activity (Navarro, Gomez et al. 2004). It was observed that some types of exercise induced an increase in muscle mass and strength, paralleled by an increase in mitochondrial content, antioxidant enzymes activity and in the synthesis of respiratory chain components (Navarro, Gomez et al. 2004; Menshikova, Ritov et al. 2006; Navarro and Boveris 2007; Paterson, Jones et al. 2007; Gomez-Cabrera, Domenech et al. 2008).

It is not surprising that mitochondria have been proposed to play a major role in aging since it is a major source of endogenously generated ROS (Boveris, Cadenas et al. 1976; Raha and Robinson 2000). Oxidative damage to the mitochondria can lead to an amplifying effect whereby damaged mitochondria release more ROS, further increasing mitochondrial oxidative damage (Harman 1972). With time, accumulation of damaged mitochondria is proposed to lead to a decrease in the capacity to produce ATP, the principal source of energy (Levine, Mosoni et al. 1996; Chang, Van Remmen et al. 2003). Thus, impaired mitochondria will contribute to age-related dysfunction

and namely a reduced aerobic capacity in muscle (Short, Bigelow et al. 2005; Figueiredo, Ferreira et al. 2008).

While other studies have been designed to evaluate the beneficial effect of moderate exercise in aging muscle, to the best of our knowledge, no work has evaluated the mitochondrial proteome alterations induced by voluntary moderate exercise in aged mice skeletal muscle and compare them with a state of absence of any physical activity. Hence, we proposed to study the effect of lifelong voluntary physical activity on mitochondrial functionality and proteome composition. To do so, we have evaluated alterations on protein regulation with 2D-PAGE, oxidative stress by assessing the protein carbonylation and functionality by determining the in-gel activity of the respiratory chain complexes. With this experimental approach we tested the hypothesis that age-related oxidative damage of proteins leads to a decline in organelle function that may be attenuated by moderate physical activity. In overall, our results provide evidence that moderate physical activity reverts some of the deleterious effects observed in sedentary aging.

2. Material and methods

2.1. Animals and Experimental Protocol

Male C57BL/6 strain mice aged two months were randomly divided into three groups (young-Y; old sedentary-S; old active-NS). After one week of quarantine, animals from Y group (n=5) were sacrificed whereas animals from S (n=5) and NS (n=5) groups were individually placed into standard cages 355x235x190 mm (Ref. 2150E, Tecniplast, Italy) and in cages equipped with running wheels (25 cm in diameter) with 364x258x350 mm (Ref. 1284L0106, Tecniplast, Italy), respectively, until sacrifice at 25 months old. NS mice performed an average of 7 Km per day during the experimental period. Animals from both groups were housed at constant temperature (21º–24°C) on a daily light schedule of 12 h of light *vs.* dark until sacrifice. All animals were provided with food and water *ad libitum*. Housing and experimental treatment of animals were in accordance with the Guide for the Care and Use of Laboratory Animals from the Institute for Laboratory Animal Research

(ILAR, 1996). The local Ethics Committee had approved the study and the experiments were complied with the current national laws.

2.2. Skeletal Muscle Mitochondria isolation

The animals were sacrificed by cervical dislocation, and hind limb muscles were extracted for preparation of isolated mitochondria, as previously described by Tonkonogi and Sahlin (1997). Briefly, muscles were immediately excised and minced in ice-cold isolation medium containing 100mM sucrose, 0.1mM EGTA, 50mM Tris-HCl, 100mM KCl, 1mM KH_2PO_4, and 0.2% BSA, pH 7.4. Minced blood-free tissue was rinsed and suspended in 10ml of fresh medium containing 0.2mg/ml bacterial proteinase (Nagarse E.C.3.4.21.62, type XXVII; Sigma) and stirred for 2 min. The sample was then carefully homogenized with a tightly fitted Potter-Elvehjen homogenizer and a Teflon pestle. After homogenization, three volumes of Nagarse-free isolation medium were added to the homogenate, which was then centrifuged at 700*g* for 10 min. The resulting supernatant suspension was centrifuged at 10,000*g* during 10 min. The supernatant was decanted, and the pellet was gently resuspended in isolation medium (1.3ml/100mg initial tissue) and centrifuged at 7,000*g* for 3 min. The final pellet, containing the mitochondrial fraction, was gently resuspended (0.4µl/mg initial tissue) in a medium containing 225mM mannitol, 75mM sucrose, 10mM Tris, and 0.1mM EDTA, pH 7.4. All mitochondrial isolation procedures were performed at 0–4°C. Mitochondrial protein concentration was spectrophotometrically estimated with the biuret method using bovine serum albumin as standard.

2.3. Identification of the proteins present in the mitochondrial proteome

Mitochondrial proteins (200µg) were solubilized in rehydration buffer and applied on an IPG strip Immobiline DryStrip pH 3-11 NL 13cm (GE Healthcare). After isoelectric focusing, the strips were prepared for the second dimension incubating in equilibration buffer with mild-agitation for 15 minutes. The second dimension was obtained on a 15% SDS-PAGE. After separation, the gels were fixed for 60 min with a 40% methanol/10% acetic acid solution

and stained overnight with colloidal coomassie. The gels were then destained with 25% methanol solution until background colour is cleared and scanned in a Bio-Rad G710 Densitometer. Digital images were analysed with PDQuest software (Bio-Rad) for matching and quantification. Protein spots were excised and tryptic in-gel digestion was performed. In brief, the gel pieces were washed twice with 25mM ammonium bicarbonate/50% acetonitrile, followed by a wash with 100% acetonitrile. They were then dried in vacuum and 25µl of 10µg/ml trypsin in 50mM ammonium bicarbonate was added to the dried residue. The samples were incubated overnight at 37°C with sequence-grade modified porcine trypsin. The tryptic peptides were extracted from the gel with formic acid and were then dried in vacuum and resuspended in 10µl of a 50% acetonitrile/0.1% formic acid solution.

2.4. Analysis of the respiratory chain complexes by BN-PAGE and in-gel activity staining

BN–PAGE was performed based on the method of Schagger and von Jagow (1991) with minor modifications. Briefly, pellet aliquots of mitochondria (400µg protein) were solubilized for 10 min on ice in 40µl of 50mM Imidazole, 1mM EDTA (pH 7.0), 50mM NaCl, 2mM 6-aminohexanoic acid, and 12µl digitonin (6.0g/g of protein) (Sigma). Proteins were separated on a 4 to 13% acrylamide–bisacrylamide BN–PAGE gel. For separation, cathode buffer (7.5mM Imidazole (pH 7.0) and 50mM tricine) containing 0.02% (w/v) Coomassie Blue G was used until the dye front had reached approximately one-third of the gel before switching for cathode buffer lacking Coomassie Blue G. Anode buffer contained 25mM Imidazole (pH 7.0). Native complexes were separated at 200V for 5 h at 4 ºC.

For identification of proteins resolved by BN-PAGE, the bands were excised and subjected to in-gel tryptic digestion as described above. For in-gel activity and histochemical staining assays of mitochondrial complexes the protocols of Zerbetto et al. (1997) were used. In brief, in-gel NADH:NTB reductase activity for complex I was determined by incubating the native gels in Complex I assay solution (2.5mg NTB and 10mL NADH (10mg/mL) were added to 1mL 5mM Tris/HCl, pH 7.4). Complex IV-specific heme stain in native

gels was determined using 10mL cytochrome *c* (5mM) and 5mg diaminobenzidine (DAB) dissolved in 1mL 50mM sodium-phosphate, pH 7.2. Complex V activity was analyzed by incubating the native gels with 35mM Tris, 270 mM glycine buffer, pH 8.3, that had been supplemented with 14mM $MgSO_4$, 0.2% (w/v) $Pb(NO_3)_2$, and 8mM ATP. Lead phosphate precipitation that is proportional to the enzymatic ATP hydrolysis activity, was stopped by 50% (v/v) methanol (30 min), and the gels were then transferred to water.

2.5. Protein identification by Maldi-Tof/Tof mass spectrometry

Mass spectra were obtained on a matrix-assisted laser desorption/ionization–time-of-flight MALDI-TOF/TOF mass spectrometer (4700 Proteomics Analyzer, Applied Biosystems, Foster City, CA, USA) in the positive ion reflector mode. A data-dependent acquisition method was created to select the five most intense peaks in each sample spot for subsequent tandem mass spectrometry (MS/MS) data acquisition, excluding those from the matrix, due to trypsin autolysis or acrylamide peaks. Trypsin autolysis peaks were used for internal calibration of the mass spectra, allowing a routine mass accuracy of more than 25ppm. Spectra were processed and analyzed by the Global Protein Server Workstation (Applied Biosystems), which uses internal Mascot software (Matrix Science, London, UK) on searching the peptide mass fingerprints and MS/MS data. Searches were performed against the Swissprot nonredundant protein database, allowing oxidation of methionine and acrylamide adducts (propionamide) of the cysteine residues as variable modifications. Positive identifications were accepted up to 95% of confidence level.

2.6. Analysis of carbonylation in mitochondrial proteins.

For protein carbonyl derivatives assay, a given volume (V) of sample containing 20µg of protein was derivatized with 2,4-dinitrophenylhydrazine (DNPH). Briefly, the sample was mixed with 1V of 12% SDS plus 2V of 20mM DNPH/10% TFA, followed by 30 minutes incubation in dark, after which 1,5V of 2M Tris-base/18.3% of β-mercaptoethanol was added for neutralization. After dilute the derivatised proteins in TBS to obtain a final concentration of 0.001µg/µL, a 100µL volume was slot-blotted into a nitrocellulose membrane.

Carbonylation was also assessed in 2-DE oxyblot as described by Conrad et al (2001). Briefly, the isoelectric focusing was carried out as described above. Then the IPG strips were derivatized with 10mM DNPH/2N TFA. After incubating for 20 minutes in dark, the reaction was stopped with 2M Tris-base/30% glycerol for 15 min. The strips were then prepared for the second dimension as described above. After separation by molecular weight, proteins were transferred for a nitrocellulose membrane.

Both slot blot and 2D-blot membranes were blocked with 5% (w/v) dry non-fat milk in TBS-T for 3 hours and then incubated overnight at 4ºC with a solution of anti-DNP antibody (Dako) in a dilution 1:2000 in blocking solution. The membranes were washed three times (10 minutes/each) with TBS-T and incubated for 2 hours with a solution of horseradish-conjugated anti-rabbit antibody (Amersham Pharmacia) in a dilution of 1:1000. Detection was carried out with enhanced chemiluminescence (Amersham Pharmacia). Quantitative analysis of slot blot was performed with Quantity One software (Bio-Rad). Mean and standard error of the mean were calculated and one-way ANOVA, followed by Tukey-Kramer multiple comparisons test were used to assess differences between groups. The level of significance was set at 5%.

3. Results

3.1. 2D-PAGE and Maldi-Tof/Tof identification of proteins from isolated mitochondria

Mitochondrial proteins were resolved by 2D-PAGE (Figure II.1). Duplicates from all the experimental groups were subject to PDQuest analysis for matching the observed spots. This analysis identified an average per gel of 233 protein spots in the Y group, 205 spots in the NS group and 263 spots in the S group. A total of 496 spots were excised from the gels and, after Maldi-Tof/Tof analysis, 273 spots were positively identified containing 79 different proteins that are the most abundant in the mitochondrial proteome. The identified proteins were grouped in 4 major classes according to their physiological function: metabolic, membrane transport, stress and other proteins (Table II.1). Most of the identified proteins belong to oxidative phosphorylation system. We have found 75 spots corresponding to 33

different proteins of the respiratory chain, mainly subunits of Complex I. This figure corresponds to almost half the proteins identified, yet it is not surprising as the respiratory chain is the main pathway within mitochondria. Other well represented metabolic subclass was the TCA cyle, with 13 proteins identified in 79 distinct spots. Additionally, 8 proteins from β-oxidation and 2 proteins from the urea cycle were detected. Besides metabolic proteins, we have also identified proteins associated with membrane transport, namely ANT 1, VDAC-1, VDAC-2 and VDAC-3. Several proteins were detected in more than one spot; considering the theoretical values of molecular weight and pI, some of these spots appeared below the expected molecular weight, suggesting they are fragments. These was more evident for aconitate hydratase (34 spots), VDAC-1 (16 spots), citrate synthase, malate dehydrogenase and ATP synthase α chain (12 spots each).

Table II.1 – List of all the identified proteins on the mitochondrial proteome, grouped in classes according to the physiological function.

Protein name and accession number	MW	pI	No. of spots
Metabolic proteins			
Respiratory chain			
(Q91VD9) NADH-ubiquinone oxidoreductase 75 kDa subunit, mitochondrial precursor (EC 1.6.5.3) (EC 1.6.99.3) (Complex I-75Kd) (CI-75Kd)	79697.53	5.51	2
(Q91YT0) NADH-ubiquinone oxidoreductase 51 kDa subunit, mitochondrial precursor (EC 1.6.5.3) (EC 1.6.99.3) (Complex I-51KD) (CI-51KD)	50801.71	8.51	2
(Q91WD5) NADH-ubiquinone oxidoreductase 49 kDa subunit, mitochondrial precursor (EC 1.6.5.3) (EC 1.6.99.3) (Complex I-49KD) (CI-49KD)	52591.64	6.52	2
(Q99LC3) NADH-ubiquinone oxidoreductase 42 kDa subunit, mitochondrial precursor (EC 1.6.5.3) (EC 1.6.99.3) (Complex I-42KD) (CI-42KD)	40577.73	7.63	3
(Q9DCT2) NADH-ubiquinone oxidoreductase 30 kDa subunit, mitochondrial precursor (EC 1.6.5.3) (EC 1.6.99.3) (Complex I-30KD) (CI-30KD)	30188.54	6.4	2
(Q8K3J1) NADH-ubiquinone oxidoreductase 23 kDa subunit, mitochondrial precursor (EC 1.6.5.3) (EC 1.6.99.3) (Complex I-23KD) (CI-23KD) (TYKY subunit)	24022.75	5.89	1
(Q9CQJ8) NADH-ubiquinone oxidoreductase B22 subunit (EC 1.6.5.3) (EC 1.6.99.3) (Complex I-B22) (CI-B22)	21838.68	7.83	1
(Q9D6J5) NADH-ubiquinone oxidoreductase ASHI subunit, mitochondrial precursor (EC 1.6.5.3) (EC 1.6.99.3) (Complex I-ASHI) (CI-ASHI)	21861.50	6.15	1

Protein name and accession number	MW	pI	No. of spots
(Q9DCS9) NADH-ubiquinone oxidoreductase PDSW subunit (EC 1.6.5.3) (EC 1.6.99.3) (Complex I-PDSW) (CI-PDSW)	20879.4	8.36	1
(Q9DCJ5) NADH-ubiquinone oxidoreductase 19 kDa subunit (EC 1.6.5.3) (EC 1.6.99.3) (Complex I-19KD) (CI-19KD) (Complex I-PGIV) (CI-PGIV)	19848.03	8.8	1
(Q7TMF3) NADH-ubiquinone oxidoreductase subunit B17.2 (EC 1.6.5.3) (EC 1.6.99.3) (Complex I-B17.2) (CI-B17.2) (CIB17.2)	17075.55	9.38	1
(Q99LY9) NADH-ubiquinone oxidoreductase 15 kDa subunit (EC 1.6.5.3) (EC 1.6.99.3) (Complex I-15 kDa) (CI-15 kDa)	12508.29	9.12	1
(Q9CQC7) NADH-ubiquinone oxidoreductase B15 subunit (EC 1.6.5.3) (EC 1.6.99.3) (Complex I-B15) (CI-B15)	14940,94	9,89	1
(Q9CPP6) NADH-ubiquinone oxidoreductase 13 kDa-B subunit (EC 1.6.5.3) (EC 1.6.99.3) (Complex I-13Kd-B) (CI-13Kd-B) (Complex I subunit B13)	13220.16	8.04	1
(Q62425) NADH-ubiquinone oxidoreductase MLRQ subunit (EC 1.6.5.3) (EC 1.6.99.3) (Complex I-MLRQ) (CI-MLRQ)/ (P29419) ATP synthase e chain, mitochondrial (EC 3.6.3.14)	9320.87 / 8099.43	9.52 / 9.34	1
(Q8K2B3) Succinate dehydrogenase [ubiquinone] flavoprotein subunit, mitochondrial precursor (EC 1.3.5.1) (Fp) (Flavoprotein subunit of complex II)	72539.17	7.06	2
(Q9CQA3) Succinate dehydrogenase [ubiquinone] iron-sulfur protein, mitochondrial precursor (EC 1.3.5.1) (Ip) (Iron-sulfur subunit of complex II)	31792.89	8.96	1
(Q9CZ13) Ubiquinol-cytochrome-c reductase complex core protein I, mitochondrial precursor (EC 1.10.2.2)	52735.42	5.75	6
(Q9DB77) Ubiquinol-cytochrome-c reductase complex core protein 2, mitochondrial precursor (EC 1.10.2.2) (Complex III subunit II)	48205.01	9.26	3
(Q9D0M3) Cytochrome c1, heme protein, mitochondrial precursor (Cytochrome c-1)	35304.94	9.24	3
(Q9CR68) Ubiquinol-cytochrome c reductase iron-sulfur subunit, mitochondrial precursor (EC 1.10.2.2) (Rieske iron-sulfur protein) (RISP)	29349.19	8.91	3
(Q9D855) Ubiquinol-cytochrome c reductase complex 14 kDa protein (EC 1.10.2.2) (Complex III subunit VI)	13387.91	9.1	2
(Q8R1I1) Ubiquinol-cytochrome c reductase complex 7.2 kDa protein (EC 1.10.2.2) (Cytochrome C1, nonheme 7 kDa protein) (Complex III subunit X) (7.2 kDa cytochrome c1-associated protein subunit)	7440.87	9.16	1
(P62898) Cytochrome c, somatic	11466.98	9.61	1
(P12787) Cytochrome c oxidase polypeptide Va, mitochondrial precursor (EC 1.9.3.1	16020.24	6.08	1
(P19536) Cytochrome c oxidase polypeptide Vb, mitochondrial precursor (EC 1.9.3.1)	13803.9	8.69	1
(P43023) Cytochrome c oxidase polypeptide VIa-heart, mitochondrial precursor (EC 1.9.3.1) (COXVIAH)	10742.41	9.24	1
(Q03265) ATP synthase alpha chain, mitochondrial precursor (EC 3.6.3.14)	59715.58	9.22	16
(P56480) ATP synthase beta chain, mitochondrial precursor (EC 3.6.3.14)	56265.46	5.19	7
(Q9DCX2) ATP synthase D chain, mitochondrial (EC 3.6.3.14)	18606.55	5.52	2

Protein name and accession number	MW	pI	No. of spots
(P97450) ATP synthase coupling factor 6, mitochondrial precursor (EC 3.6.3.14) (ATPase subunit F6)	12488.59	9.36	2
(Q99LC5) Electron transfer flavoprotein alpha-subunit, mitochondrial precursor (Alpha-ETF)	35017.51	8.62	1
(Q9DCW4) Electron transfer flavoprotein beta-subunit (Beta-ETF)	27292.82	8.57	1
TCA Cycle			
(Q99KI0) Aconitate hydratase, mitochondrial precursor (EC 4.2.1.3) (Citrate hydro-lyase) (Aconitase)	85410.02	8.08	34
(Q9CZU6) Citrate synthase, mitochondrial precursor (EC 2.3.3.1)	51703.37	8.72	12
(O08749) Dihydrolipoyl dehydrogenase, mitochondrial precursor (EC 1.8.1.4) (Dihydrolipoamide dehydrogenase)	54178.12	7.97	4
(Q01205) Dihydrolipoyllysine-residue succinyltransferase component of 2-oxoglutarate dehydrogenase complex, mitochondrial precursor (EC 2.3.1.61) (Dihydrolipoamide succinyltransferase component of 2-oxoglutarate dehydrogenase complex) (E2) (E2K)	47382.64	8.17	1
(P97807) Fumarate hydratase, mitochondrial precursor (EC 4.2.1.2) (Fumarase) (EF-3)	54336.08	9.12	1
(Q64521) Glycerol-3-phosphate dehydrogenase, mitochondrial precursor (EC 1.1.99.5) (GPD-M) (GPDH-M)	80848.42	6.17	1
(P54071) Isocitrate dehydrogenase [NADP], mitochondrial precursor (EC 1.1.1.42) (Oxalosuccinate decarboxylase) (IDH) (NADP+-specific ICDH) (IDP) (ICD-M)	58711.76	8.89	2
(P08249) Malate dehydrogenase, mitochondrial precursor (EC 1.1.1.37)	35573.75	8.83	12
(Q60597) 2-oxoglutarate dehydrogenase E1 component, mitochondrial precursor (EC 1.2.4.2) (Alpha-ketoglutarate dehydrogenase)	116043.2	6.51	4
(P35486) Pyruvate dehydrogenase E1 component alpha subunit, somatic form, mitochondrial precursor (EC 1.2.4.1) (PDHE1-A type I)	43203.62	8.49	1
(Q9D051) Pyruvate dehydrogenase E1 component beta subunit, mitochondrial precursor (EC 1.2.4.1) (PDHE1-B)	38912.01	6.41	3
(Q9Z2I9) Succinyl-CoA ligase [ADP-forming] beta-chain, mitochondrial precursor (EC 6.2.1.5) (Succinyl-CoA synthetase, betaA chain) (SCS-betaA) (ATP-specific succinyl-CoA synthetase beta subunit)	50082.12	6.57	2
(Q9D0K2) Succinyl-CoA:3-ketoacid-coenzyme A transferase 1, mitochondrial precursor (EC 2.8.3.5) (Somatic-type succinyl CoA:3-oxoacid CoA-transferase) (Scot-S)	55953	8.73	1
β-oxidation			
(Q61425) Short chain 3-hydroxyacyl-CoA dehydrogenase, mitochondrial precursor (EC 1.1.1.35) (HCDH) (Medium and short chain L-3-hydroxyacyl-coenzyme A dehydrogenase)	34441.87	8.76	1
(P51174) Acyl-CoA dehydrogenase, long-chain specific, mitochondrial precursor (EC 1.3.99.13) (LCAD)	47877.48	8.53	6
(P45952) Acyl-CoA dehydrogenase, medium-chain specific, mitochondrial precursor (EC 1.3.99.3) (MCAD)	46451.62	8.6	1
(Q9QYR9) Acyl coenzyme A thioester hydrolase, mitochondrial precursor (EC 3.1.2.2) (Very-long-chain acyl-CoA thioesterase) (MTE-I)	49620.71	6.91	1
(Q8QZT1) Acetyl-CoA acetyltransferase, mitochondrial precursor (EC 2.3.1.9) (Acetoacetyl-CoA thiolase)	44787.33	8.71	1

Protein name and accession number	MW	pI	No. of spots
(P52825) Carnitine O-palmitoyltransferase II, mitochondrial precursor (EC 2.3.1.21) (CPT II)	73880.47	8.46	1
(P47934) Carnitine O-acetyltransferase (EC 2.3.1.7) (Carnitine acetylase) (CAT) (Carnitine acetyltransferase) (CrAT)	70879.31	8.52	1
(Q61425) Short chain 3-hydroxyacyl-CoA dehydrogenase, mitochondrial precursor (EC 1.1.1.35) (HCDH) (Medium and short chain L-3-hydroxyacyl-coenzyme A dehydrogenase)	34441.87	8.76	2
Urea cycle			
(Q8CHT0) Delta-1-pyrroline-5-carboxylate dehydrogenase, mitochondrial precursor (EC 1.5.1.12) (P5C dehydrogenase) (Aldehyde dehydrogenase 4A1)	61771.57	8.58	1
(P05202) Aspartate aminotransferase, mitochondrial precursor (EC 2.6.1.1) (Transaminase A) (Glutamate oxaloacetate transaminase-2)	47381.21	9.13	3
Membrane Transport			
(P48962) ADP,ATP carrier protein, heart/skeletal muscle isoform T1 (ADP/ATP translocase 1) (Adenine nucleotide translocator 1) (ANT 1) (Solute carrier family 25, member 4) (mANC1)	32752,09	9,73	1
(Q60932) Voltage-dependent anion-selective channel protein 1 (VDAC-1) (mVDAC1) (mVDAC5) (Outer mitochondrial membrane protein porin 1) (Plasmalemmal porin)	32331.38	8.55	16
(P81155) Voltage-dependent anion-selective channel protein 2 (VDAC-2) (Outer mitochondrial membrane protein porin 2) (B36-VDAC)	31725.63	7.44	1
(Q60931) Voltage-dependent anion-selective channel protein 3 (VDAC-3) (mVDAC3) (Outer mitochondrial membrane protein porin 3)	30733.38	8.96	2
Stress proteins			
(Q9QZ58) Heat-shock protein beta-3 (HspB3)	17198.94	5.12	1
(P09671) Superoxide dismutase [Mn], mitochondrial precursor (EC 1.15.1.1)	24587.5	8.8	2
Others			
(Q9D172) ES1 protein homolog, mitochondrial precursor	28072.77	9	1
(P32848) Parvalbumin alpha	11792,05	5,02	2
(P30275) Creatine kinase, ubiquitous mitochondrial precursor (EC 2.7.3.2) (U-MtCK) (Mia-CK) (Acidic-type mitochondrial creatine kinase)	46974.19	8.39	1
(P09605) Creatine kinase, sarcomeric mitochondrial precursor (EC 2.7.3.2) (S-MtCK) (Mib-CK) (Basic-type mitochondrial creatine kinase)	47355.30	8.76	11
(Q8CAQ8) Mitochondrial inner membrane protein (Mitofilin)	83848.20	6.18	1
(O55126) NipSnap2 protein (Glioblastoma amplified sequence)	32911.85	9.31	2
Not previously described in mitochondria			
(P07310) Creatine kinase, M chain (EC 2.7.3.2) (M-CK)	43017.80	6.58	2
(P53569) CCAAT/enhancer binding protein zeta (CCAAT-box-binding transcription factor) (CCAAT-binding factor) (CBF)	113758.7	5.45	1
(Q8BV13) COP9 signalosome complex subunit 7b (Signalosome subunit 7b) (SGN7b) (JAB1-containing signalosome subunit 7b)	29670.43	5.93	1
(P02092) Hemoglobin beta chain	15662.69	10.0	2
(P02564) Myosin heavy chain, cardiac muscle beta isoform (MyHC-beta)	222944.6	5.64	1

Protein name and accession number	MW	pI	No. of spots
(Q9D6Z1) Nucleolar protein Nop56 (Nucleolar protein 5A)	64424.10	9.22	2
(P11157) Ribonucleoside-diphosphate reductase M2 chain (EC 1.17.4.1) (Ribonucleotide reductase small chain)	45066.78	5.33	2
(P07724) Serum albumin precursor	68647.71	5.75	1
(Q8R164) Valacyclovir hydrolase precursor (EC 3.1.-.-) (VACVase) (Biphenyl hydrolase-like protein)	32830.14	9.06	1

Comparing the 2-DE gels from different tested groups, a common profile between the three groups was observed (Figure II.1). From the PDQuest analysis of the gels from the three test groups, 24 spots had a match within all the gels (Figure II.1-A). Grouping the matched spots by their physiological function, 9 contained proteins from the respiratory chain, 7 contained proteins from the TCA cycle, 5 were from creatine kinase and the remainder spot was from Mn-SOD. In one spot, two distinct proteins, Complex I subunit MLRQ and ATP synthase e chain, were identified by Maldi-Tof/Tof analysis. These proteins have similar values of pI and MW, and thus it is not odd, however, quantitative analysis cannot distinguish between the two.

The quantitative PDQuest analysis of the identified protein spots, with a match in all gels, points to an up-regulation of some respiratory chain proteins in S mice, while TCA proteins are down-regulated (Table II.2). For example, Complex I PDSW subunit, Complex III iron-sulphur subunit and ATP synthase D chain increased in the S group. Yet, the ATP synthase coupling factor 6 presented a different variation when compared to Y group; while there was no differences between Y and S groups, this protein was down-regulated in the NS group. Complex IV subunits and ATP synthase α chain did not present any variation among groups. Regarding the TCA cycle proteins, while aconitate hydratase was up-regulated in the S group, citrate synthase almost doubled for the NS group. The spots containing fragments of these proteins were found generally diminished in the S group. Parvalbumin α was up-regulated in the S group, not showing differences in the NS group. Mn-SOD was found up-regulated in the NS group and did not present differences in the S group.

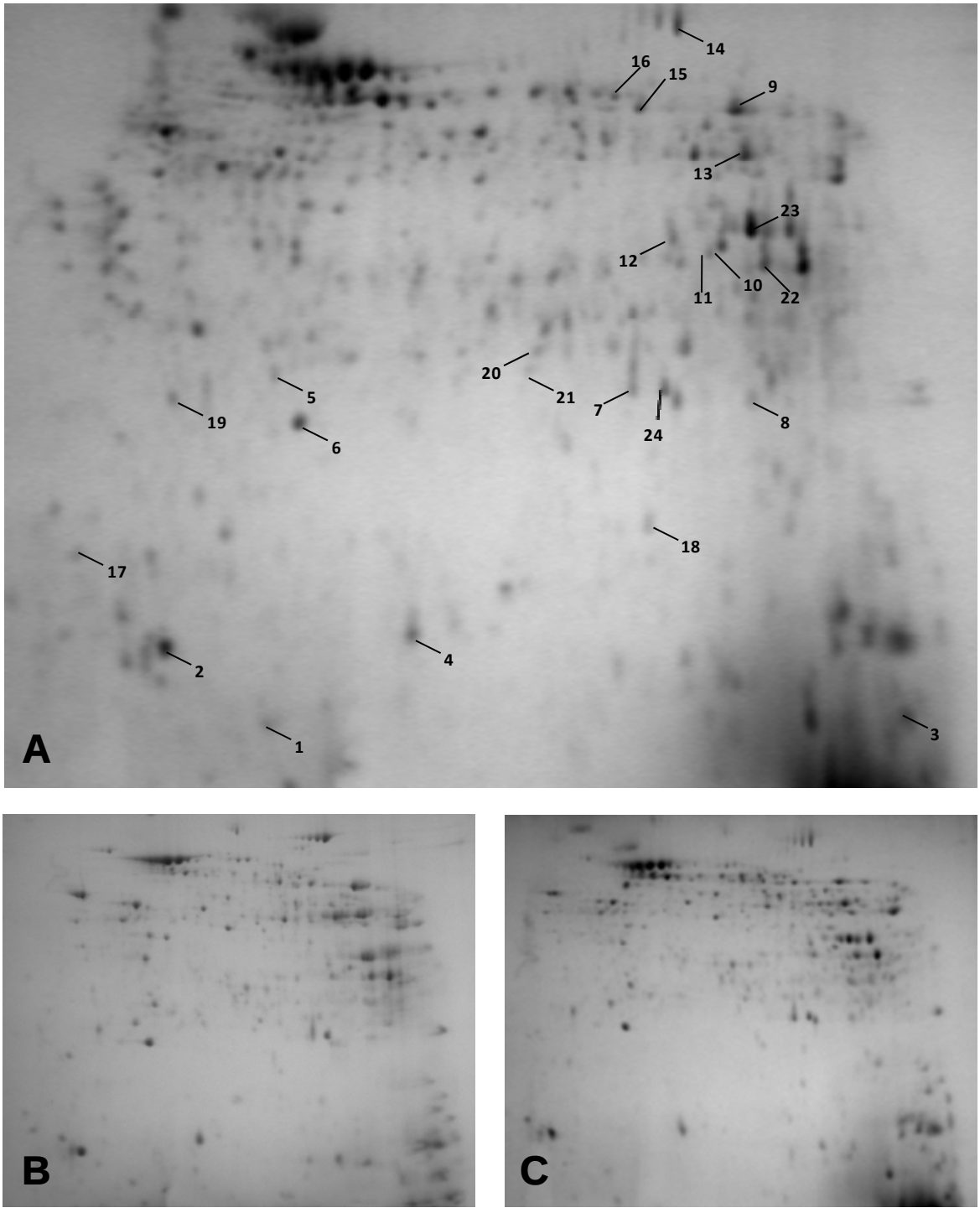


Figure II.1 – Representative 2D-PAGE from isolated mitochondria of all the test groups. Panel A: Young mice; Panel B: Non-sedentary mice; Panel C – Sedentary mice. For numbering refer to Table II.2

Table II.2 – Proteins with a match on the three groups and their relative abundance. (#) – spot considered as a fragment. Spot's intensity compared to the Y group: (O) – no difference found; (-) – down-regulated; (+) – up-regulated. The number of symbols is proportional to the increase / decrease found.

Spot no.		Protein Name	NS	S
1		(P97450) ATP synthase coupling factor 6, mitochondrial precursor (EC 3.6.3.14) (ATPase subunit F6)	-	O
2		(P12787) Cytochrome c oxidase polypeptide Va, mitochondrial precursor (EC 1.9.3.1)	O	O
3		(Q62425) NADH-ubiquinone oxidoreductase MLRQ subunit (EC 1.6.5.3) (EC 1.6.99.3) (Complex I-MLRQ) (CI-MLRQ) / (P29419) ATP synthase e chain, mitochondrial (EC 3.6.3.14)	++	-
4		(P19536) Cytochrome c oxidase polypeptide Vb, mitochondrial precursor (EC 1.9.3.1)	O	O
5		(Q9DCX2) ATP synthase D chain, mitochondrial (EC 3.6.3.14)	O	+
6	#	(Q9DCX2) ATP synthase D chain, mitochondrial (EC 3.6.3.14)	O	O
7		(Q9CR68) Ubiquinol-cytochrome c reductase iron-sulfur subunit, mitochondrial precursor (EC 1.10.2.2) (Rieske iron-sulfur protein) (RISP)	O	+
8		(Q9DCS9) NADH-ubiquinone oxidoreductase PDSW subunit (EC 1.6.5.3) (EC 1.6.99.3) (Complex I-PDSW) (CI-PDSW)	O	+
9		(Q03265) ATP synthase alpha chain, mitochondrial precursor (EC 3.6.3.14)	O	O
10	#	(Q99KI0) Aconitate hydratase, mitochondrial precursor (EC 4.2.1.3) (Citrate hydro-lyase) (Aconitase)	O	--
11	#	(Q99KI0) Aconitate hydratase, mitochondrial precursor (EC 4.2.1.3) (Citrate hydro-lyase) (Aconitase)	O	---
12	#	(Q9CZU6) Citrate synthase, mitochondrial precursor (EC 2.3.3.1)	-	-
13		(Q9CZU6) Citrate synthase, mitochondrial precursor (EC 2.3.3.1)	+	O
14		(Q99KI0) Aconitate hydratase, mitochondrial precursor (EC 4.2.1.3) (Citrate hydro-lyase) (Aconitase)	O	+
15	#	(Q99KI0) Aconitate hydratase, mitochondrial precursor (EC 4.2.1.3) (Citrate hydro-lyase) (Aconitase)	O	O
16	#	(Q99KI0) Aconitate hydratase, mitochondrial precursor (EC 4.2.1.3) (Citrate hydro-lyase) (Aconitase)	-	O
17		(P32848) Parvalbumin alpha	O	++
18	#	(P09605) Creatine kinase, sarcomeric mitochondrial precursor (EC 2.7.3.2) (S-MtCK) (Mib-CK) (Basic-type mitochondrial creatine kinase)	O	-
19	#	(P09605) Creatine kinase, sarcomeric mitochondrial precursor (EC 2.7.3.2) (S-MtCK) (Mib-CK) (Basic-type mitochondrial creatine kinase)	++	++
20	#	(Q60932) Voltage-dependent anion-selective channel protein 1 (VDAC-1) (mVDAC1) (mVDAC5) (Outer mitochondrial membrane protein porin 1) (Plasmalemmal porin)	O	O
21	#	(P09605) Creatine kinase, sarcomeric mitochondrial precursor (EC 2.7.3.2) (S-MtCK) (Mib-CK) (Basic-type mitochondrial creatine kinase)	O	O
22		(Q9Z2L0) Voltage-dependent anion-selective channel protein 1 (VDAC-1) (rVDAC1) (Outer mitochondrial membrane protein porin 1)	O	O
23	#	(P09605) Creatine kinase, sarcomeric mitochondrial precursor (EC 2.7.3.2) (S-MtCK) (Mib-CK) (Basic-type mitochondrial creatine kinase)	O	-
24		(P09671) Superoxide dismutase [Mn], mitochondrial precursor (EC 1.15.1.1)	+	O

3.2. Assessment of the carbonyl content

To assess the levels of protein oxidation we have measured the total carbonyl content by slot blot and with 2-DE oxyblot we identified the proteins more affected by carbonylation. The data from the quantitative analysis of slot blot was normalized to the amount of protein loaded.

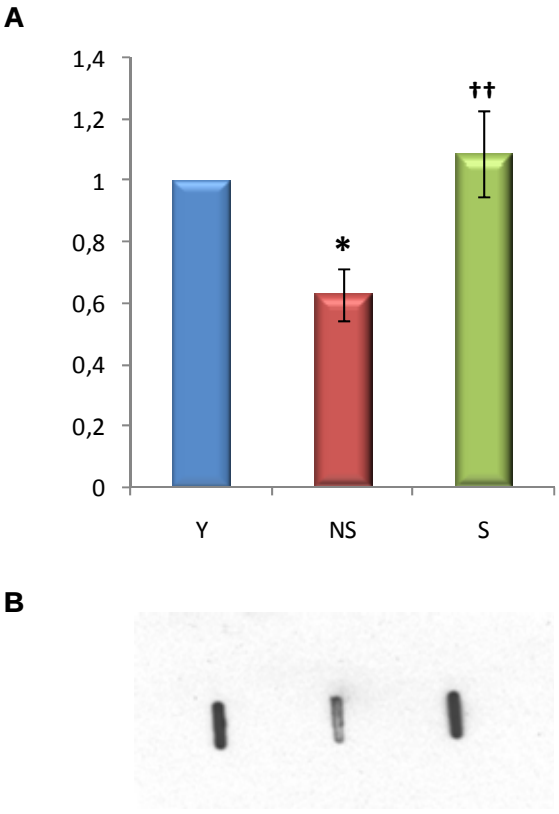


Figure II.3 – Panel A: Carbonyl content determined by slot blot. Data are presented as the fold-increase versus the young group ±SEM from two individuals of each group in four independent experiments. (* - p<0,05 for NS vs. Y; †† - p<0,01 for S vs. NS). Panel B: Representative image of the slot blot.

Carbonyl content was found decreased in NS and increased in S mice, when compared to Y mice. As can be observed in Figure II.3, significantly lower total carbonyl content was present in NS mice. Moreover, S mice presented a very significant increase in protein carbonylation when compared to NS. These results were corroborated by the 2-DE oxyblots (Figure II.4).

A closer analysis of 2-DE oxyblots, showed a clear prevalence of carbonylated proteins in S mice (Figure II.4-C). While some proteins were found carbonylated in the three groups, such as, ATP synthase α chain, citrate synthase, VDAC-1 and fragments of aconitate hydratase and creatine kinase

(Figure II.4), other proteins were only found carbonylated in NS and S mice. These latter include Complex III iron-sulphur subunit; Mn-SOD, aspartate aminotransferase, creatine kinase, malate dehydrogenase and some distinct spots of aconitate hydratase and creatine kinase fragments (Figures II.4-B, II.4-C). Furthermore, some spots corresponding to ATP synthase D chain, Complex I MLRQ subunit or ATP synthase e chain (both proteins were identified on the same spot) and cytochrome *c* and cytochrome c_1 were only found carbonylated on S mice (Figure II.4-C). A group of spots corresponding to intact aconitate hydratase was only detected in the 2-DE oxyblot of Y and S mice, being absent from the 2-DE oxyblot of NS mice (Figure II.4-B).

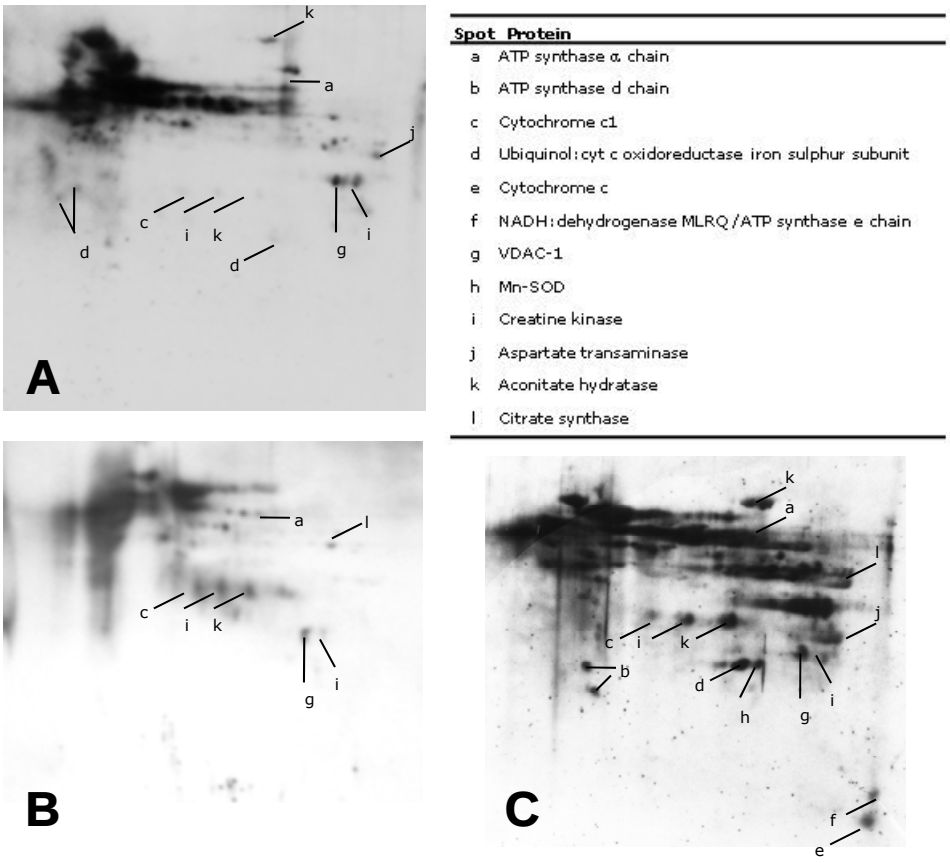


Figure II.4 – Representative 2-DE oxyblots of each group and identification of the most significant carbonylated proteins; A – young mice, B – non-sedentary mice, C – sedentary mice;

3.3. In-gel activity of respiratory chain complexes

To assess mitochondrial functionality in the three groups, we have determined the in-gel activity of the respiratory Complexes I, IV and V resolved by BN-PAGE. Differences in the activity between experimental groups were observed (Figure II.5). Comparing the activity of Complexes IV and V to the Y group, there was an increase in the NS group, while it markedly decreased in the S group. Contrastingly, Complex I activity presented no differences among groups.

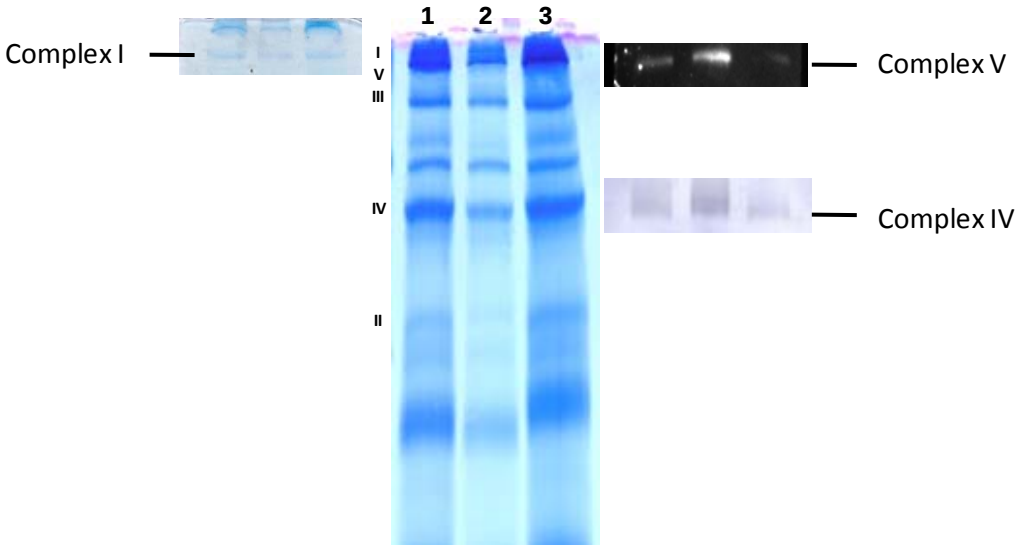


Figure II.5 – BN-PAGE and histochemical staining of complexes from the respiratory chain to evaluate their in-gel activity. Centre figure is representative of a BN-PAGE gel stained with colloidal coomassie. On the left is a representative image of the complex I staining, on the right are representative images of complexes IV and V staining. Lane 1 – young mice; lane 2 – non-sedentary mice; lane 3 – sedentary mice.

4. Discussion

Progressive mitochondrial dysfunction has been implicated in the aging process. This has led to interest in comparative mitochondrial proteomics, to analyze the changes behind the age-related functional decline. A great deal of information is already known about the proteins that populate mitochondria and the metabolism that occurs within them. However, on a protein composition point of view, mitochondria are quite complex and encompass both very soluble proteins (present in the matrix and the intermembrane space) and very hydrophobic membrane proteins, plus membrane proteins of

intermediate solubility, such as some subunits of the oxidative phosphorylation complexes or the outer membrane porins (Rabilloud 2008). Recent studies have increased the number of mitochondrial proteins known in muscle (Sickmann, Reinders et al. 2003; Zhang, Li et al. 2008). Nonetheless, not only the effects of aging in the mitochondrial proteome are not fully unveiled, but the role of lifelong physical activity is even more undisclosed. Thus, this study represents the first comprehensive analysis of the effect of lifelong physical activity on the mitochondrial proteome and function. The mitochondrial proteome of young, non-sedentary old and sedentary old C57BL/6 mice was resolved by 2-DE followed by Maldi-Tof/Tof identification (Figure II.1 and Table II.2). The comparative PDQuest analysis suggested an up-regulation of most OXOPHOS proteins in sedentary old mice, as well as aconitate hydratase and parvalbumin. In fact, a previous study has already demonstrated that skeletal muscle mitochondrial proteins are up-regulated in 18 months rats (Chang, Cornell et al. 2007). Hence, proteomic results suggest lifestyle as a key modulator factor, since lifelong moderate physical activity kept expression levels of these proteins at young mice levels, overcoming the results obtained with caloric restriction (Chang, Cornell et al. 2007).

Our results from 2-DE also showed that the levels of Mn-SOD were up-regulated in non-sedentary mice compared to young and sedentary mice (Table II.2). Previous observations that reported an elevation of antioxidant enzyme activities in aged skeletal muscle (Leeuwenburgh, Fiebig et al. 1994; Vasilaki, McArdle et al. 2006). Radák et al. (2002) also reported an increase in the activity of Mn-SOD with aging in *gastrocnemius* muscle fibres; however the Mn-SOD content remained similar. Moderate exercise has been reported to increase the basal levels and activity of antioxidant enzymes (Leeuwenburgh, Fiebig et al. 1994; Leeuwenburgh and Heinecke 2001; Sastre, Pallardo et al. 2003; Niess and Simon 2007). Nonetheless, some authors have reported that exercise does not seem to offer additional protection against oxidative stress in senescent muscle (Leeuwenburgh, Fiebig et al. 1994; Vasilaki, McArdle et al. 2006).

These results from Mn-SOD regulation suggest an increased protection against oxidative stress in NS mice, which is further confirmed by the carbonyl

content determined by slot blot (Figure II.3). Indeed, protein carbonyl content is a widely used marker for protein oxidation and is generally accepted that aging increases protein susceptibility to oxidative damage (Stadtman 2001; Stadtman and Levine 2003). Indeed, increased oxidative stress from progressive mitochondrial dysfunction is considered a basic mechanism of mammalian aging, as proposed by the free radical theory of aging. However, regarding the carbonyl content, contradictory studies have been reported. In an already mentioned study, Radák et al (2002) reported no significant differences in total carbonyl content in *gastrocnemius* myofibers from aged rat. In our study, the total carbonyl content also did not show significant differences between S and Y mice; nonetheless, NS mice presented a significant decrease in the total carbonyl content compared to Y mice, and a very significant decrease compared to S mice (Figure II.3-A). It has been suggested that the exercise-induced antioxidant and housekeeping enzymes activity, overcomes the increase in ROS production. (Goto, Nakamura et al. 1999; Choksi and Papaconstantinou 2008; Radak, Chung et al. 2008). Thus, our results from the carbonyl content and Mn-SOD up-regulation in NS mice, provide further evidence of a protector role of lifelong physical activity against protein oxidative damage.

Although the results from slot blot do not suggest an aggravation of oxidative stress with aging, the 2-DE oxyblots from Y and S mice suggest an increase in protein oxidative damage for this latter (Figure II.4-C). When we identify the proteins more prone to age-related oxidative damage, we found preferentially carbonylated Complex III iron-sulphur subunit, aspartate aminotransferase, creatine kinase, malate dehydrogenase and some distinct spots of aconitate hydratase and creatine kinase fragments (Figure II.4). Similar observations were reported by Prokai et al. (2007) and Chung et al. (2008) that identified target proteins for age-related carbonylation in rat brain and heart mitochondria, respectively. These include ATP synthase β subunits, VDAC-1 and ANT-2, in addition to those observed in this study. Our data also suggest a protective modulator role of lifelong activity since in NS group there was a clear decrease in the intensity and number of carbonylated spots compared to S group (Figure II.4). Moreover, proteins such as Complex I

MLRQ subunit/ATP synthase e chain, cytochrome *c* and cytochrome c_1 only appeared carbonylated in S group (Figure II.5-C). This is in opposition to Choksi and Papaconstatinou (2008) that did not observe a consistent age-related carbonylation susceptibility of ETC components, which was justified by the interaction of other unspecified factors.

Several proteins were present in more than one spot, pointing to fragmentation. Indeed, protein oxidation can result in cleavage of the polypeptide backbone (Stadtman 2006). Given that the proteins present in these displaced spots (Table II.2) were also identified to be prone to oxidative damage by the 2-DE oxyblots analysis, it is clear that aconitate hydratase, creatine kinase, ATP synthase D chain and citrate synthase are susceptible to fragmentation (Figure II.4 and Table II.2). Surprisingly, more than 30 spots with aconitate hydratase were identified in 2-DE gels from sedentary mice, being some of them carbonylated, which points to oxidative induced fragmentation.

So, we have already demonstrated that lifelong physical activity keeps protein expression level, at old age, similar to young individuals' level and decreases oxidative stress. To determine how these finding could be reflected on mitochondrial functionality, we evaluated the in-gel activity of the respiratory chain complexes.

It is described that aging increases oxidative damage on the respiratory chain complexes from mice and consequently has a negative impact on mitochondrial functionality (Navarro, Gomez et al. 2005; Choksi, Nuss et al. 2007; Choksi and Papaconstantinou 2008). Moreover, oxidative modifications may elicit protein misfolding stress response that contributes to mitochondrial dysfunction in aged mice (Choksi and Papaconstantinou 2008). Also, previous work has already demonstrated that moderate exercise may invert this tendency namely by preventing the reduction of Complexes I and IV activity (Navarro, Gomez et al. 2005). Indeed, the results presented herein corroborate this notion. As can be depicted from the analysis of Figure II.5, a noticeable decrease in the in-gel activity of Complexes IV and V in sedentary mice is noticed compared to Y group, while in non-sedentary mice there is an increase of both complexes activity. Unexpectedly, the activity of complex I

did not present noticeable differences among experimental groups. In overall, our in-gel activity results are matching with the carbonyl content profile and further confirm that physical activity attenuate the age-related decreased activity of the respiratory chain, particularly Complex IV and V.

In conclusion, our results strongly suggest a protective role of lifelong moderate physical activity, once the loss of mitochondrial functionality was significantly attenuated in comparison to sedentary lifestyle. Moreover, non-sedentary mice had even shown a reduction of protein oxidative damage when compared to young and sedentary old individuals. Furthermore, sedentarism was shown to up-regulate some of the respiratory chain proteins in skeletal muscle mitochondrial proteome, which is probably in an underlying mechanism, although not successful, to overcome the loss of functionality induced by age-related oxidative damage.

CHAPTER III – Oxidative modifications on Complex I of the Electron Transport Chain

III. OXIDATIVE MODIFICATIONS ON COMPLEX I OF THE ELECTRON TRANSPORT CHAIN

1. Introduction

Being Complex I one of the sites for electron leakage and superoxide production on the electron transport chain (ETC) (Loschen, Flohe et al. 1971; Boveris, Oshino et al. 1972; Choksi and Papaconstantinou 2008), it must be particularly susceptible to oxidative modifications (Murray, Taylor et al. 2003). Some of the subunits of Complex I have already been described as oxidatively modified. Murray et al (2003) have assessed the *in vitro* modification of tyrosine residues to 3-nitrotyrosine by peroxynitrite, in some subunits of Complex I and validated their data with tandem mass spectrometry. Three subunits of Complex I were found to have nitrated tyrosines, specifically, the α subcomplex subunit 12 (B17.2 subunit), the β subcomplex subunit 4 (B15 subunit) and the α subcomplex subunit 6 (B14 subunit). Additionally, it was also shown the oxidation of one tryptophan and one methionine residues in the B17.2 subunit (Murray, Taylor et al. 2003). Regarding mitochondrial proteins, few studies provided *in vivo* results. Taylor et al. (2003) have identified several proteins susceptible to double oxidation of tryptophan residues in the mitochondrial proteome of human and bovine heart. Within Complex I subunits analyzed, the most susceptible appeared to be the flavoprotein 1 subunit (51 kDa) and the alpha subcomplex subunit 9 (39 kDa subunit). Each one of these subunits presented three double oxidized residues of tryptophan. Additionally, double oxidized residues of tryptophan were also found on other subunits of Complex I, namely, five iron-sulphur subunits (75kDa, 30kDa, 23kDa, 20kDa and 18kDa) and the α subcomplex subunit 5 (13 kDa-B subunit) (Taylor, Fahy et al. 2003). Another *in vivo* study was carried by Meany et al (2007) using avidin affinity chromatography to isolate carbonylated proteins from rat skeletal muscle mitochondria, previously biotinylated. They have found modified peptides from 94 mitochondrial proteins 15 of which belonging to Complex I. The distribution of the

carbonylated subunits was apparently random within the Complex I structure (Meany, Xie et al. 2007).

Under exercise, skeletal muscle is certainly one of the tissues with higher demands of ATP; accordingly it has a large, although variable, number of mitochondria, depending on the specific needs. For instance, aerobic exercise was shown to increase the number of mitochondria (Wright 2007). Age and exercise have been reported to increase the oxidant production in skeletal muscle (Bejma and Ji 1999). Moreover, the aging process is traditionally associated with an increase in the free-radical production by the ETC and in the damage to cellular components (Bejma and Ji 1999; Stadtman 2006).

To address the targets of oxidation in the mitochondrial Complex I, we have isolated mitochondria from the hind limbs of mice. Complex I subunits were analyzed by tandem mass spectrometry to identify precisely the type and location of the modification. We have observed that tryptophan was the most susceptible amino acid to oxidative modification. Our results also suggest that non-catalytic subunits are more tolerant to oxidative damage and catalytic subunits may be promptly replaced in case of damage. Additionally, we assessed the extent of the oxidative modification of these proteins with aging and how the sedentary vs. non-sedentary lifestyle may modulate it.

2. Material and Methods

2.1. Animals and Experimental Protocol

Male C57BL/6 strain mice aged two months were randomly divided into three groups (young-Y; old sedentary-S; old non-sedentary-NS). After one week of quarantine, animals from Y group were sacrificed whereas animals from S and NS groups were individually placed into standard cages 355x235x190 mm (Ref. 2150E, Tecniplast, Italy) and in cages with 364x258x350 mm (Ref. 1284L0106, Tecniplast, Italy) equipped with running wheels (25 cm in diameter), respectively, until sacrifice at 25 months old. Animals from both groups were housed at constant temperature (21º–24°C) on a daily-light schedule of 12 h of light vs. dark until sacrifice. All animals were provided with food and water *ad libitum*. Housing and experimental

treatment of animals were in accordance with the Guide for the Care and Use of Laboratory Animals from the Institute for Laboratory Animal Research (ILAR, 1996). The local Ethics Committee had approved the study and the experiments were complied with the current national laws.

2.2. Skeletal Muscle Mitochondria isolation

The animals were sacrificed by cervical dislocation, and hind limb muscles were extracted for preparation of isolated mitochondria, as previously described by Tonkonogi and Sahlin (1997). Briefly, muscles were immediately excised and minced in ice-cold isolation medium containing 100mM sucrose, 0.1mM EGTA, 50mM Tris-HCl, 100mM KCl, 1mM KH_2PO_4, and 0.2% BSA, pH 7.4. Minced blood-free tissue was rinsed and suspended in 10ml of fresh medium containing 0.2mg/ml bacterial proteinase (Nagarse E.C.3.4.21.62, type XXVII; Sigma) and stirred for 2 min. The sample was then carefully homogenized with a tightly fitted Potter-Elvehjen homogenizer and a Teflon pestle. After homogenization, three volumes of Nagarse-free isolation medium were added to the homogenate, which was then centrifuged at 700*g* for 10 min. The resulting supernatant suspension was centrifuged at 10,000*g* during 10 min. The supernatant was decanted, and the pellet was gently resuspended in isolation medium (1.3ml/100mg initial tissue) and centrifuged at 7,000*g* for 3 min. The final pellet, containing the mitochondrial fraction, was gently resuspended (0.4µl/mg initial tissue) in a medium containing 225mM mannitol, 75mM sucrose, 10mM Tris, and 0.1mM EDTA, pH 7.4. All mitochondrial isolation procedures were performed at 0–4°C. Mitochondrial protein concentration was spectrophotometrically estimated with the biuret method using bovine serum albumin as standard.

2.3. Two-dimensional polyacrylamide gel electrophoresis

Proteins from the mitochondrial (200µg) fraction were solubilised in rehydration buffer and applied on an IPG strip Immobiline DryStrip pH 3-11 NL 13cm (GE Healthcare). After isoelectric focusing, the second dimension was obtained on a 15% SDS-PAGE. Following separation by molecular weight, the gels were fixed in 40% methanol/10% acetic acid for 1 hour and stained with

colloidal coomassie overnight. Destaining was achieved with 30 minutes washes with 25% methanol until the background colour was removed. Gels were scanned in a Bio-Rad G710 Densitometer. Digital images were analysed with PDQuest software (Bio-Rad) for matching and quantification.

2.4. Sample preparation for analysis by mass spectrometry

Protein spots of were excised and tryptic in-gel digestion was performed. In brief, the gel pieces were washed twice with 25mM ammonium bicarbonate/50% acetonitrile, followed by a wash with 100% acetonitrile. They were then dried in vacuum and 25μl of 10μg/ml trypsin in 50mM ammonium bicarbonate was added to the dried residue. The samples were incubated overnight at 37°C with sequence-grade modified porcine trypsin. The tryptic peptides were extracted from the gel with formic acid and were then dried and resuspended in 10μl of a 50% acetonitrile/0.1% formic acid solution.

2.5. Protein identification by Maldi-Tof/Tof mass spectrometry

Mass spectra were obtained on a matrix-assisted laser desorption/ionization–time-of-flight MALDI-TOF/TOF mass spectrometer (4700 Proteomics Analyzer, Applied Biosystems, Foster City, CA, USA) in the positive ion reflector mode. A data-dependent acquisition method was created to select the five most intense peaks in each sample spot for subsequent tandem mass spectrometry (MS/MS) data acquisition, excluding those from the matrix, due to trypsin autolysis or acrylamide peaks. Trypsin autolysis peaks were used for internal calibration of the mass spectra, allowing a mass accuracy of more than 25ppm. Spectra were processed and analyzed by the Global Protein Server Workstation (Applied Biosystems), which uses internal Mascot software (Matrix Science, London, UK) on searching the peptide mass fingerprints and MS/MS data. Searches were performed against the Swissprot nonredundant protein database, allowing oxidation of methionine and acrylamide adducts (propionamide) of the cysteine residues as variable modifications. Positive identifications were accepted up to 95% of confidence level.

2.6. Identification and confirmation of oxidative modifications by Maldi-Tof/Tof mass spectrometry

Protein spots identified as subunits of Complex I that had a match in the three groups tested were analysed once more by the Global Protein Server Workstation (Applied Biosystems). This time the search allowed as variable modifications the oxidative modifications listed in the UNIMOD website. To ensure the accuracy of the matches, only peptides with a match error below 30ppm were accepted. The m/z values of the precursor ions, which the software analysis for protein identification detected as being modified, were fragmented in a collision cell using air as the collision gas. The resultant spectra were once more processed by the Global Protein Server Workstation (Applied Biosystems) and the results that confirmed the previous analysis were further analysed in the DeNovo Explorer (Applied Biosystems) for manual confirmation of the modifications.

3. Results

A common 2-DE profile was observed among different experimental groups. From the identification by Maldi-Tof/Tof of excised spots, seven spots identified as subunits of Complex I had a clear match in all the three groups observed (Figure III.1).

The matched spots contained proteins from distinct areas of the Complex I. Three of these proteins belong to the peripheral arm of the Complex I protruding towards the matrix, being one of them part of the flavoprotein portion. The other four proteins identified in the three test groups belong to the membrane arm (Figure III.2).

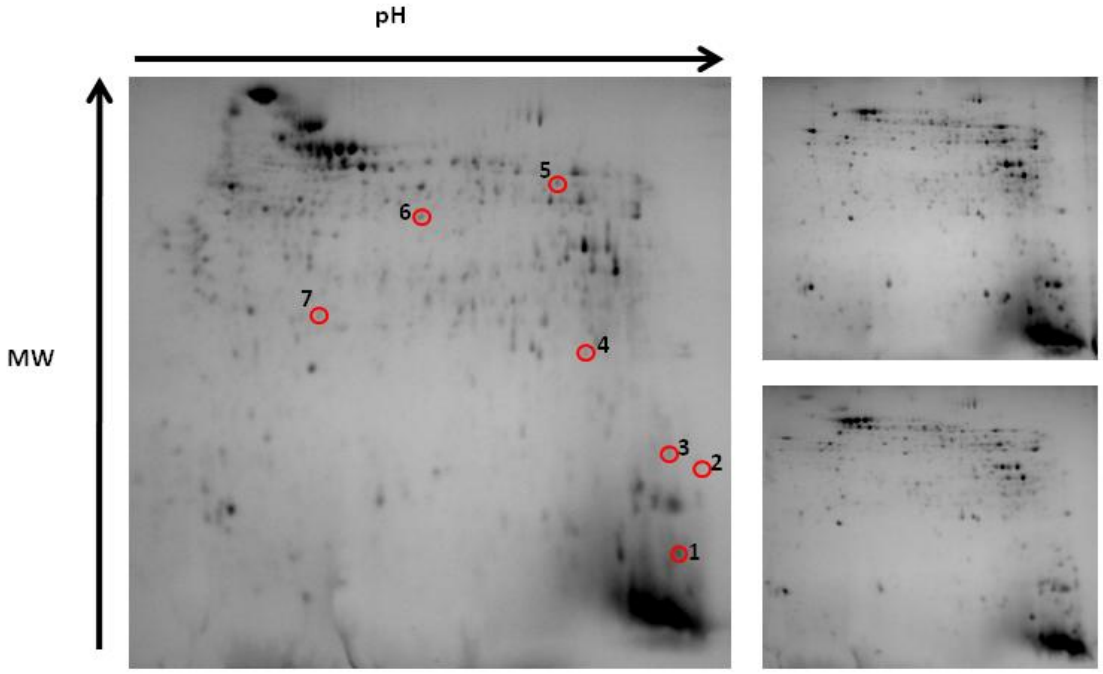


Figure III.1 – Representative images of skeletal muscle mitochondrial proteome, resolved by 2D-PAGE, from the three test groups. On the left is the Y mice gel, upper right is the NS mice and lower right is the S mice gels. For the numbering, refer to Table III.2.

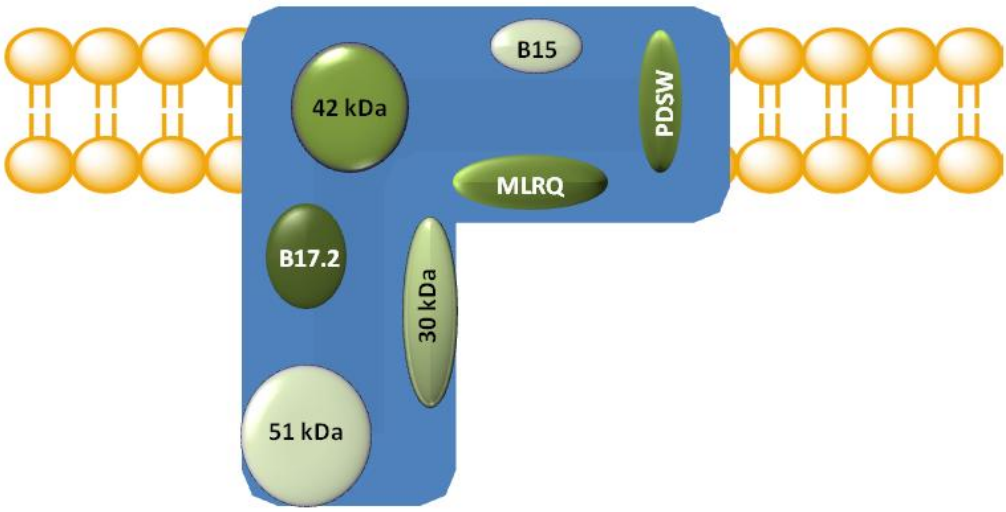


Figure III.2 – Schematic representation of complex I and location of the analyzed subunits within it. The intensity of the color is proportional to the overall ratio of modified/unmodified peptides.

A comparative quantitative analysis using PDQuest was carried out and it was observed that the old mice skeletal muscle mitochondrial proteome is enriched in subunits from complex I. Two subunits do not follow this pattern. Subunit MLRQ is up-regulated in NS mice and down-regulated in S mice; subunit B15 is down-regulated in NS mice, although it is up-regulated in S mice (Figure III.3-A). The relative percentage of each subunit within the set of proteins analyzed also changed among the test groups. There was a slight increase in the amount of the 30 kDa subunit in the NS and S mice. NS mice also presented a skeletal muscle mitochondrial proteome enriched in B17.2 subunit, compared to the others. Yet, the B15 subunit is extremely decreased in the NS mice, when compared with the other groups. The MLRQ subunit appeared to be enriched in the NS mice, decreasing on the Y mice and being only one third of the amount on the S mice.

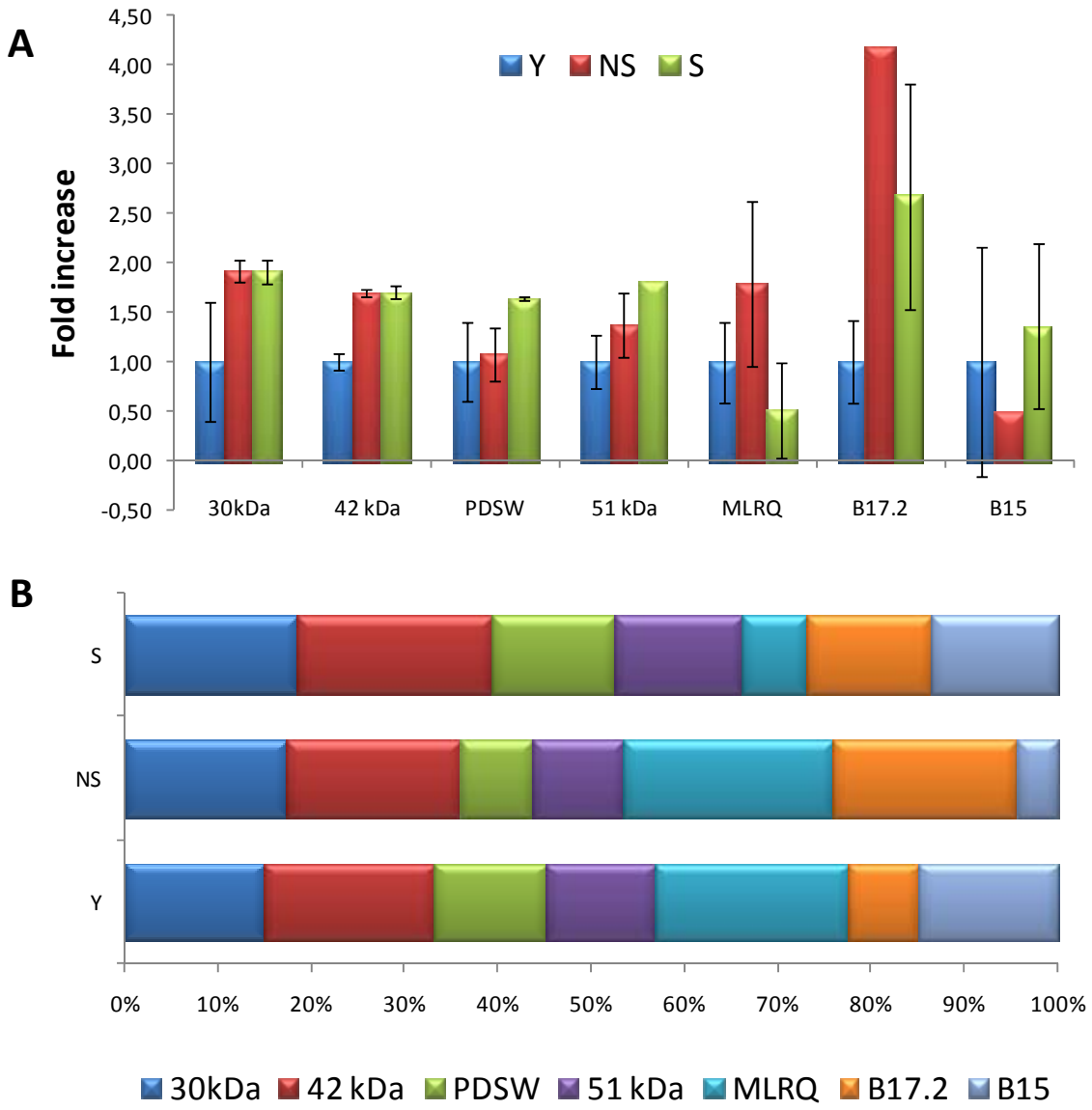


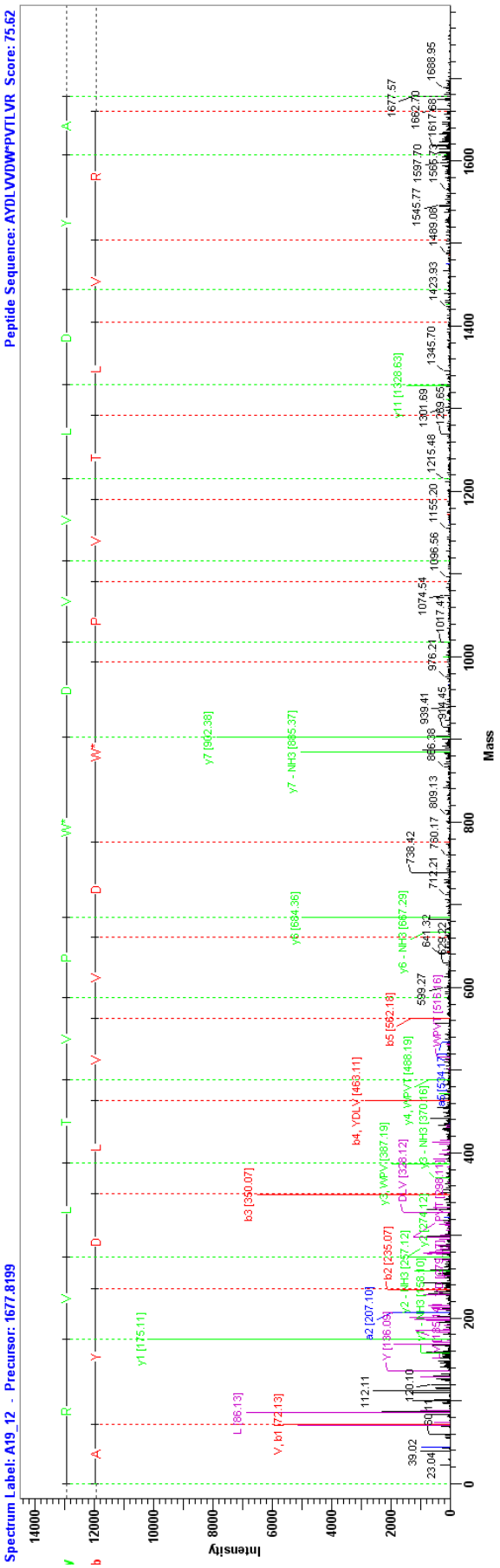
Figure III.3 – Relative amounts of each subunit related to the young mice group. Panel A – Fold increase of the amount; Panel B –Relative amount of each subunit within the group of subunits analyzed for each test group. Y – young mice, NS – non-sedentary mice; S – sedentary mice.

Following this comparative analysis, these 7 proteins were selected for further analysis/search for the oxidative modifications listed in Table III.1.

Table III.1 – Oxidative modifications listed on UNIMOD web site that were searched for with GPS workstation

Modification	Description	Mass shift (monoisotopic)
Amino (Tyr)	Tyrosine oxidation to 2-aminotyrosine	+15.0108
Arg->GluSA	Arginine oxidation to glutamic semialdehyde	-43.0534
Carbamylation (Lys, Arg, Cys, Met)	Isocyanate reaction with amino groups	+43.0058
Carboxy (Lys, Asp, Glu)	Carboxylation	+43.9898
Cys->oxoalanine	Cysteine oxidation to oxoalanine	-17.9928
Deamidation (Arg, Asn, Gln)	Deamidation	0.9840
His->Asn	Histidine oxidation to asparagine	-23.0159
His->Asp	Histidine oxidation to aspartic acid	-22.0319
Lys->Allysine	Lysine oxidation to aminoadipic semialdehyde	-1.0316
Lys->Aminoadipic acid	Lysine oxidation to α-aminoadipic acid	+14.9632
Oxidation and dioxidation	Oxygen addition and hydroxylation	+15.9949 +31.9898
Pro->Pyro-Glu	Proline oxidation to pyroglutamic acid	+13.9792
Pro->Pyrrolidinone	Proline oxidation to pyrrolydinone	-30.0105
Pro->Pyrrolidone	Proline oxidation to pyrrolidone	-27.9949
Quinone (Tyr)	Quinone	+29.9741
Trioxidation (Cys)	Cysteine oxidation to cysteic acid	+47.9847
Trp->Hydroxykynurenin	Tryptophan oxidation to hydroxykynurenin	+19.9898
Trp->Kynurenin	Tryptophan oxidation to kynurenin	+3.9949
Trp->Oxolactone	Tryptophan oxidation to oxolactone	+13.9792

The GPS workstation search detected several oxidative modifications on these subunits, which were then confirmed with tandem mass spectrometry, to identify precisely which modification was present and which residue was modified (Figure III.4). More than 550 MS/MS spectra were acquired resulting in the confirmation of 13 residues with modifications. Once MS/MS spectra allowed the confirmation of the modification, it was assumed that if the same precursor ion of that spectrum was present in any spectrum of that protein, it was from the same peptide with the same modification, not requiring additional confirmation.


Peptide Sequence: AYDLVVDW*PVTLVR Score: 75.62
Spectrum Label: A19_12 - Precursor: 1677.8199
Intensity
Mass
y7 [902.38]
y7 - NH3 [885.37]
y6 [684.36]
y6 - NH3 [667.29]
y11 [1328.63]
y1 [175.11]
b3 [350.07]
b4, YDLV [463.11]
b5 [562.18]
L [86.13]
V, b1 [72.13]
a2 [207.10]
b2 [235.07]

(Previous page)

Figure III.4 – Representative MS/MS spectrum after the analysis with DeNovo Explorer software. The fragmentation of the ion with m/z=1677.8199 revealed it was the peptide AYDLVVDWPVTLVR from the PDSW subunit, where the residue W presented a mass shift of +32 Da, corresponding to the oxidation to NFK. In red are the mass peaks corresponding to b ions and in green the mass peaks corresponding to the y ions.

Tryptophan appeared to be the most susceptible amino acid as 7 of the 13 modifications identified were in tryptophan residues. This residue presented two states of oxidation: single oxidation to hydroxytryptophan (HTRP) and double oxidation to N-formylkynurenin (NFK), with mass shifts of +16 and +32 Da, respectively (Figure III.5).

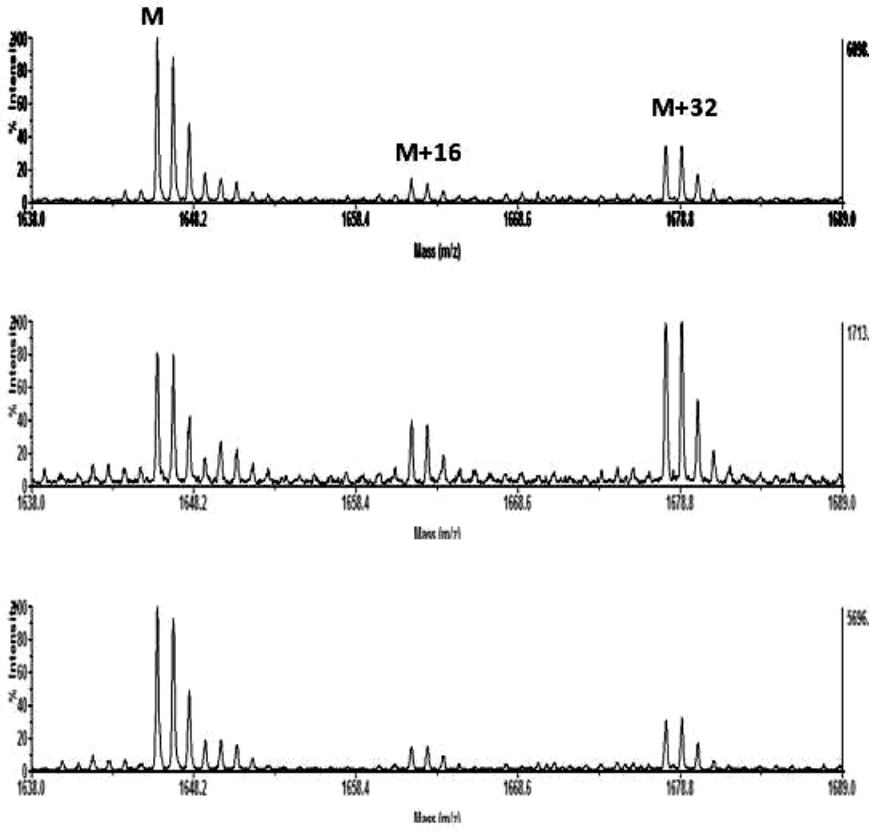


Figure III.5 – Representative Maldi-Tof/Tof mass spectrum where it is clearly visible the mass shift of +16 and +32 Da relating to the unmodified peptide (M). The spectra are from PDSW subunit on the three tested groups. From top to down: young, non-sedentary aged and sedentary aged mice.

Table III.2 summarizes the modifications found and their location within the primary structure. The protein with more confirmed oxidative modifications was the PDSW subunit, while the 51 kDa and B15 subunits had no modifications confirmed. All the detected modifications were present in the three test groups, although with different degrees of extent. For each modification we have found the mass peaks corresponding to the unmodified peptide and to different degrees of modification in the MS spectrum. To compare the relative abundance of each modification, we have calculated the ratio between the intensities of the mass peak of the modified peptide and the unmodified peptide for each modification and also for the sum of the intensities of modified peptides and the unmodified peptide.

Table III.2– List of the Complex I subunits identified; in brackets is the corresponding swissprot accession number. For each subunit the peptides identified are listed, as well as the modified residue in lower case on the peptide sequence. The numbering refers to the amino acid sequence of the protein precursor listed in swissprot.

Spot	Subunit (Accession no.)	Tryptic peptide	Residue
1	MLRQ (Q62425)	LALFNPDVSwDR	W^{45}
2	B15 (Q9CQC7)	No modified peptides detected	
3	B17.2 (Q7TMF3)	WVIYTTEmnGK	N^{69}
			$N^{69}+M^{68}$
		NTFwDVDGSMVPPEWHR	W^{75}
4	PDSW (Q9DCS9)	VDQEImNIIQER	M^{102}
		AYDLVVDwPVTLVR	W^{42}
		pDSwDKDVYPEPPSR	W^{4}
			$W^{4}+P^{1}$
5	51 kDa (Q91YT0)	No modified peptides detected	
6	42 kDa (Q99LC3)	LQSwLYASR	W^{134}
		QDDwTFHYLR	W^{289}
7	30 kDa (Q9DCT2)	VVAEpVELAQEFR	P^{222}
		KFDLNSPwEAFPAYR	W^{238}
		FDLNSPwEAFPAYR	
		ILTdYGFEGHPFR	D^{189}

On the MLRQ subunit we have confirmed the oxidation of Trp^{45} to HTRP and NFK. A smaller proportion of modified peptides was found in the S and NS mice; however, the relative proportions of each modification remained similar, prevailing the oxidation to NFK (Figure III.6).

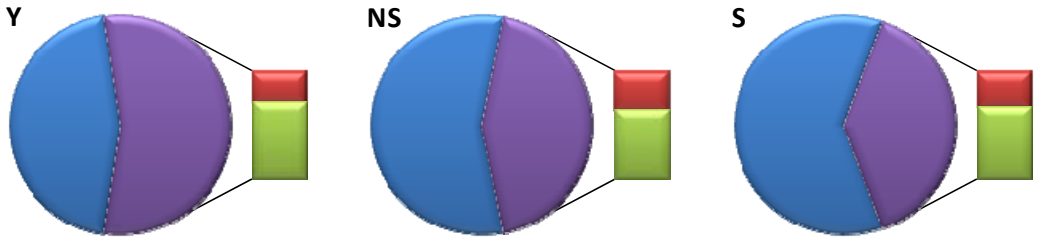


Figure III.6 – Modification on W^{45} in the MLRQ subunit. The relative proportion of unmodified (blue) vs. modified (purple) peptides is plotted on the pie charts. The contribution of each modification is presented on the lateral column: red – oxidation to HTRP; green – oxidation to NFK.

On the B17.2 subunit, we have detected three different residues modified, which are closely located in the primary structure. The peptides with an oxidation of Met^{68} and a simultaneous deamidation of Asn^{69} are more frequent than the corresponding unmodified peptide. Moreover, the NS mouse has proportionally more modified peptides than his counterparts. We have also found this peptide singly modified with a deamidation in Asn^{69}. Comparing both single and double modified peptides, it is noticeable that the presence of peptides with oxidized Met^{68} simultaneously with deaminated Asn^{69} prevails over the deaminated Asn^{69} peptides in both Y and NS mice. However, in the S mouse, the both kinds of peptides are present in equal amounts (Figure III.7-A). Additionally it was detected a modification on W^{75}. The modification of this residue was more frequent in the Y mouse, reducing frequency in the NS mouse and even more in S mouse. A closer look to the extent of the modification on the residue, shows that the single oxidation to HTRP prevailed over the double oxidation to NFK and this latter was more present in the NS mouse (Figure III.7-B).

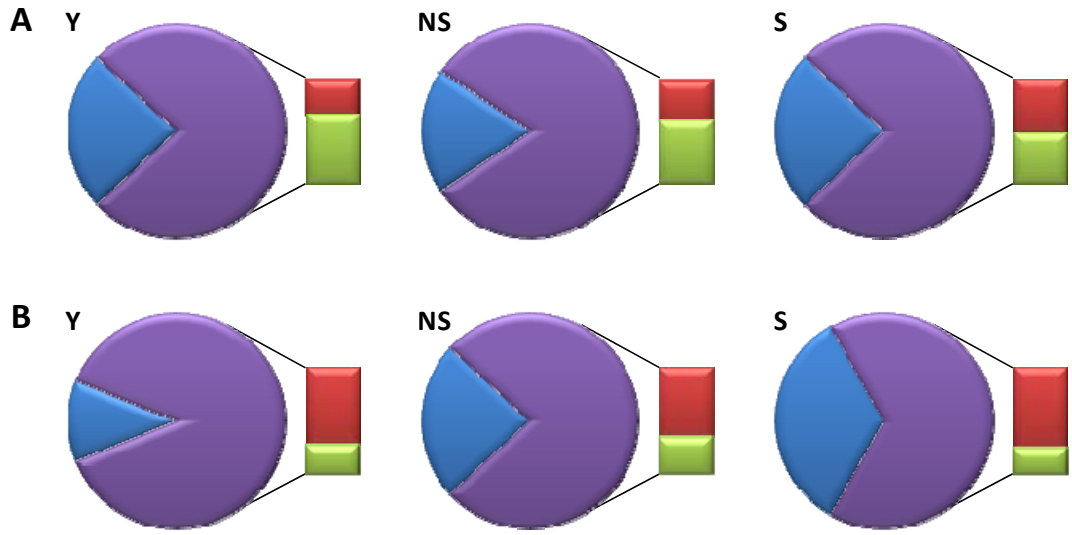


Figure III.7 – Modification on the B17.2 subunit. The relative proportion of unmodified (blue) vs. modified (purple) peptides is plotted on the pie charts. The contribution of each modification is presented on the lateral column. Panel A: red – deamidation of Asn^{69}; green – oxidation of Met^{69} and deamidation of Asn^{69}. Panel B: red – oxidation of W^{75} to HTRP; green – oxidation of W^{75} to NFK.

The PDSW subunit was one of the subunits that presented more modifications with a total of 4 modified residues, which are distributed throughout the primary structure. On its N-terminal residues Pro^{1} and Trp^{4} were found modified. The presence of modified peptides was more visible on the Y mouse, decreasing in the NS mouse and being almost equivalent to the non-modified peptides in the S mouse. Considering the extent of the modifications we have detected three differently modified peptides, namely with an oxidation of Trp^{4} to HTRP, a double oxidation of the same residue to NFK and a simultaneous oxidation of Trp^{4} to HTRP and of Pro^{1} to pyroglutamic acid. The modified peptides were evenly distributed in the three groups, with a slight prevalence of the oxidation of Trp^{4} to HTRP in the NS mouse and of the oxidation of Trp^{4} to HTRP simultaneously with the oxidation of Pro^{1} in the S mouse (Figure III.8-A). Other modified residue was the Trp^{42}. This was found oxidized to HTRP and double oxidized to NKF. The modification of this residue was more evident in the NS mouse. Considering the extent of the oxidation, all the groups presented a prevalence of the double oxidized peptides over the single oxidized, which was, again, more evident in the NS mouse (Figure

III.8-B). The last residue found modified in this protein was Met^{102}. The oxidation of this residue was very pronounced in the Y mouse, followed by the NS mouse and finally the S mouse. The double oxidation of Met^{102} was found at very low frequency in the three groups (Figure III.8-C).

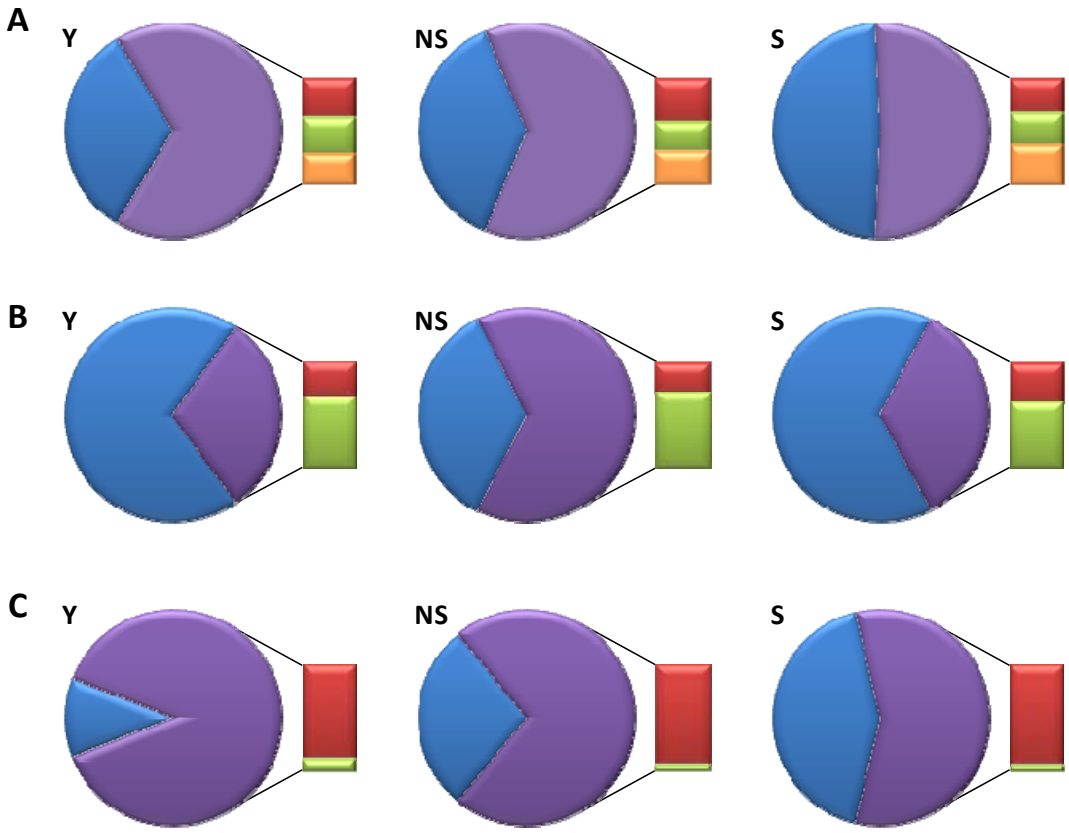


Figure III.8 – Modification on the PDSW subunit. The relative proportion of unmodified (blue) vs. modified (purple) peptides is plotted on the pie charts. The contribution of each modification is presented on the lateral column. Panel A: red – oxidation of Trp^4 to HTRP; green – oxidation of Trp^4 to NFK; orange – oxidation of Trp^4 to HTRP and oxidation of Pro^1 to pyroglutamic acid. Panel B: red – oxidation of Trp^{42} to HTRP; green – oxidation of Trp^{42} to NFK; Panel C - red – oxidation of Met^{102}; green – double oxidation of Met^{102}.

The 30 kDa subunit presented three different residues modified. The Asp^{189} residue was found oxidized and double oxidized. Modified peptides were scarce, especially in the NS and S mice. Regarding the extent of the oxidation in the residue, the single oxidation of Asp^{189} prevails over its double oxidation in all groups (Figure III.9-A). Pro^{222} was also found oxidized to glutamic semialdehyde and to pyroglutamic acid. The modified peptides were more

frequent in the Y mouse than in the NS and S mice. Moreover, the Y mouse presented more peptides oxidized to glutamic semialdehyde, whereas the S mouse only presented peptides modified to pyroglutamic acid (Figure III.9-B). The other modified peptide was Trp^{238}. This residue was found in two different tryptic peptides (Table III.2) and with two degrees of oxidation: oxidized to HTRP and double oxidized to NFK. Once more, the modified peptides are more frequent in the Y mouse, prevailing over the non-modified peptides, and less frequent in the S mouse. The three groups tested presented a prevalence of the double oxidized peptide over the single oxidized (Figure III.9-C).

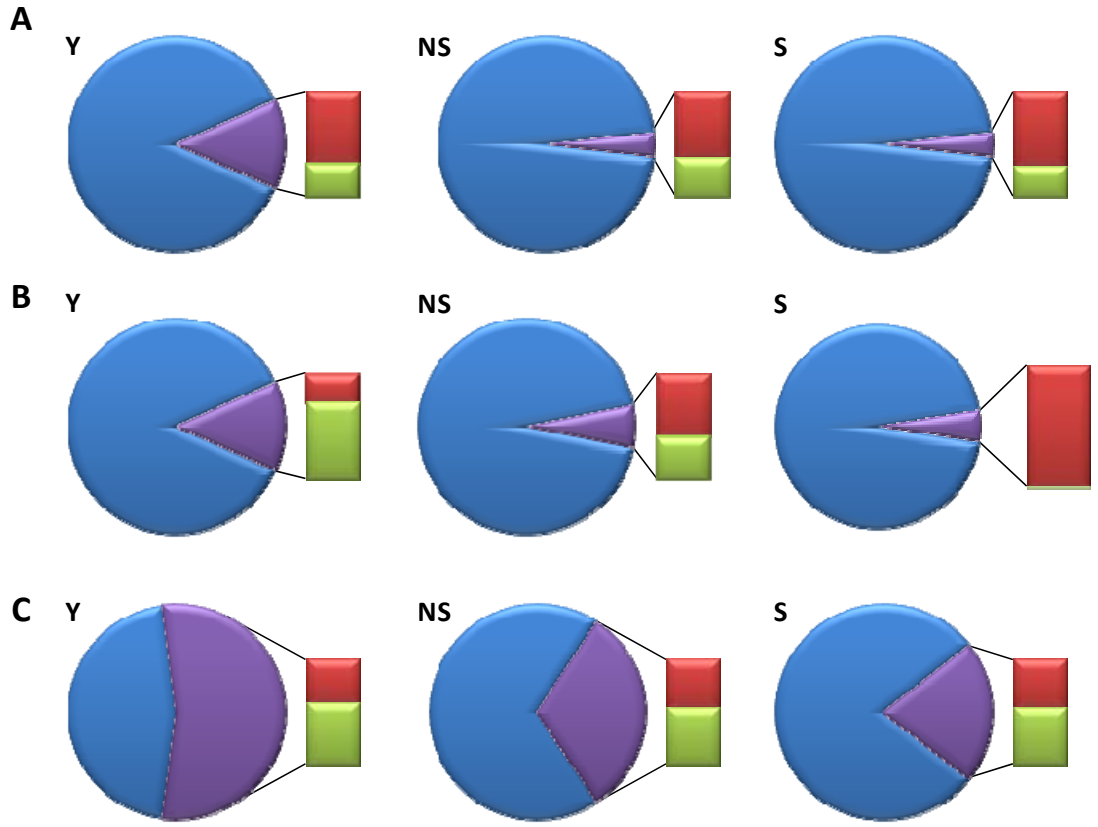


Figure III.9 – Modification on the 30kDa subunit. The relative proportion of unmodified (blue) vs. modified (purple) peptides is plotted on the pie charts. The contribution of each modification is presented on the lateral column. Panel A: red – oxidation of Asp^{189}; green – double oxidation of Asp^{189}. Panel B: red – oxidation of Pro^{222} to pyroglutamic acid; green – oxidation of Pro^{222} to glutamic semialdehyde. Panel C: red – oxidation of Trp^{238} to HTRP; green – oxidation of Trp^{238} to NFK.

The other protein with confirmed modifications was the 42kDa subunit. Two tryptophan residues were found oxidized, Trp^{134} and Trp^{289}. Both residues presented a similar pattern, however, the oxidation of Trp^{289} was more pronounced. Modified peptides were more frequent in the S mouse, prevailing over the unmodified peptide for Trp^{289}. Regarding the kind of modification detected; the double oxidation to NFK overcame the single oxidation to HTRP in all the test subjects (Figure III.10).

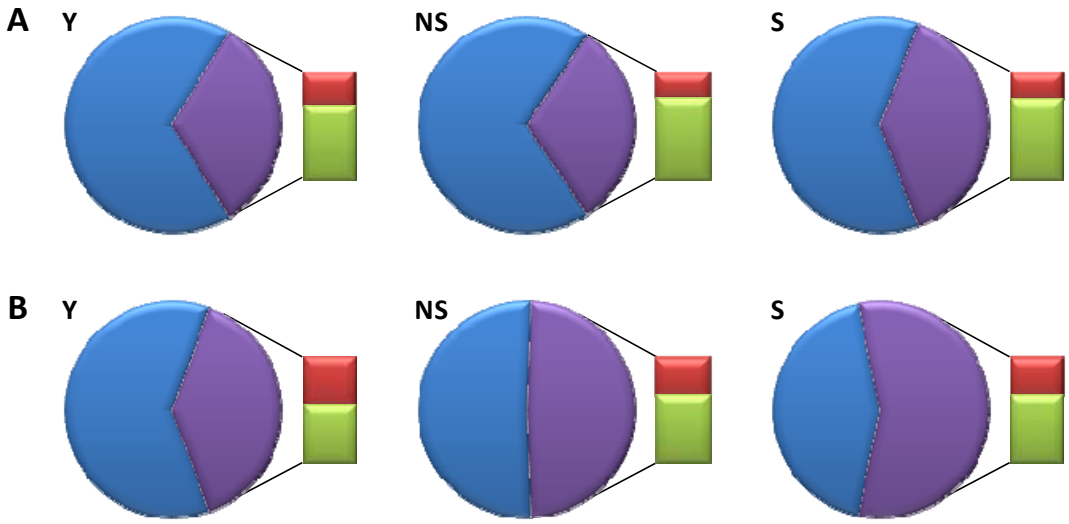


Figure III.10 – Modification on the 42kDa subunit. The relative proportion of unmodified (blue) vs. modified (purple) peptides is plotted on the pie charts. The contribution of each modification is presented on the lateral column. Panel A: red – oxidation of Trp^{134} to HTRP; green – oxidation of Trp^{134} to NFK. Panel B: red – oxidation of Trp^{289} to HTRP; green – oxidation of Trp^{289} to NFK.

Two subunits, B15 and 51kDa, did not present confirmed modifications. Also, the confirmed modifications for the other subunits were present in all the test groups. Nonetheless, the Maldi-MS spectra presented clear differences among groups, for the same protein (Figure III.11).

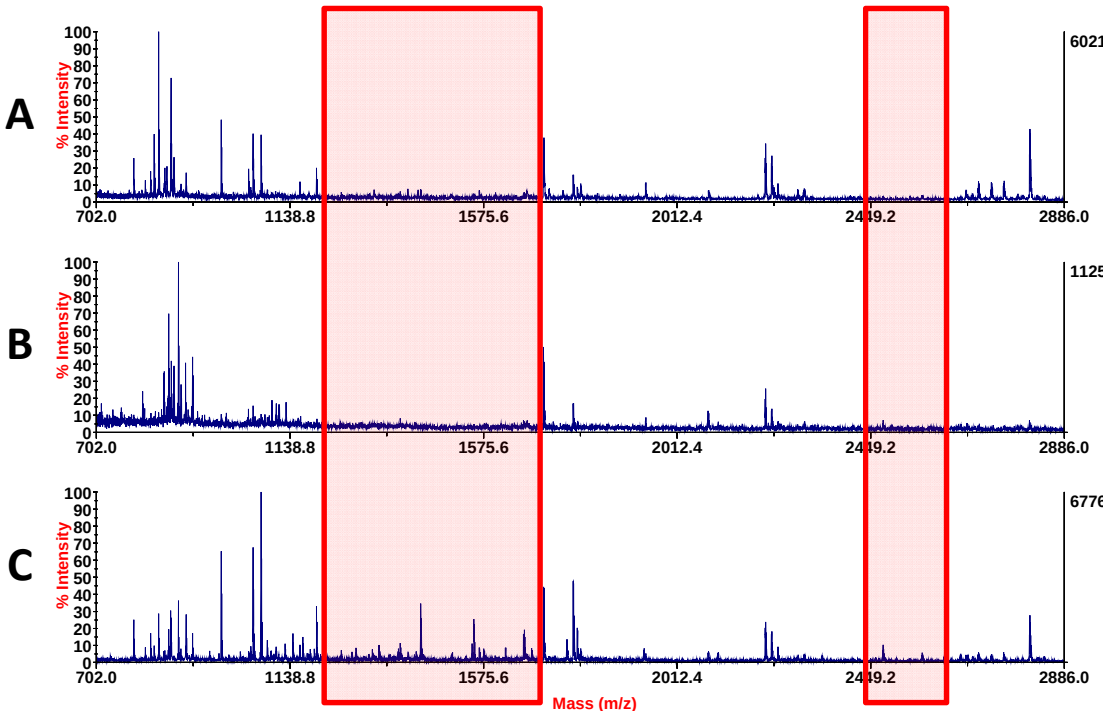


Figure III.11 – Representative Maldi MS spectra from subunit 51kDa. No modification was confirmed by MS/MS for this subunit; however, the spectra present clear differences. Panel A: Y mouse; Panel B: NS mouse; Panel C – S mouse.

4. Discussion

The post-translational modification of subunits in Complex I may result in its inactivation, thus, the identification of the modifications that may be critical for the assembly of the complex and the maintenance of its catalytic activity, as well as the site where they occur, is essential to fully understand the implications of oxidative stress in mitochondrial age-associated dysfunction (Munoz, Fernandez-Irigoyen et al. 2008). The recent advances in proteomic techniques have allowed the identification of many post-translational modifications on several proteins. Despite that, the characterization of a single protein by MS implies the analysis of hundreds of peptide variants (Nielsen, Savitski et al. 2006). In the present study we have looked for modifications induced by oxidative stress in seven identified subunits of Complex I, which are distributed through the entire Complex, according to the structural organization proposed by Brandt (2006) (Figure III.2) The theoretical values of MW and pI, obtained from the Mascot

database, corresponded to the observed in the 2D-PAGE, allowing us to conclude that intact proteins, rather than fragments, were analyzed.

Some studies have reported that the Complex I activity is decreased in aged individuals (Choksi, Nuss et al. 2007; Navarro and Boveris 2007). However, we have previously observed that Complex I activity did not present differences among the three groups (see Chapter II). As already discussed in Chapter II, this may be the result of an underlying mechanism in which the lack of functionality is overcome by the up-regulation of proteins (Figure III.3A), although it has not been successful for other ETC complexes.

The analysis of the more than 550 MS/MS spectra have resulted in the unequivocal confirmation of 13 modified residues within the subunits analyzed, the majority were tryptophan residues. Tryptophan oxidation in proteins has been reported for more than 40 years (Previero, Coletti-Previero et al. 1967). Finley et al. (1998) have studied the oxidation of tryptophan by metal-catalyzed oxidation (MCO) in bovine lens and have identified several oxidation states and their corresponding mass shifts by MS/MS. Accordingly, tryptophan can present itself in 4 different states, beyond the non-modified residue (186 Da): hydroxytryptophan (HTRP), N-formylkynurenine (NFK), kynurenin (KYN) and 3-hyroxykynurenine (3HO-KYN), with mass shifts of +16, +32, +4 and +20 Da, respectively (Finley, Dillon et al. 1998). We have observed the mass shifts corresponding to the first two modifications here described (Table III.2).

Subunit MLRQ presented Trp^{45} modified. This residue was found in two oxidation states, oxidized to HTRP and double oxidized to NFK. Unexpectedly, aged mice presented less modified peptides than young mice. Moreover, the double oxidation to NFK was more common as the ratios of NFK/unmodified peptide were almost double of the HTRP/unmodified peptide (Figure III.6).

Subunit B17.2 presented two modified peptides. One of the peptides was observed twice, one time with a deamidated Asn and another time with the deamidated Asn together with an oxidized Met. Methionine oxidation is usually considered an artefact attributed to the sample handling, in particular to the electrophoresis (Froelich and Reid 2008). Nonetheless, the oxidation on this specific residue has already been reported by Murray et al (Murray, Taylor

et al. 2003), although their results came from peroxinitrite-induced oxidations. Moreover, other authors have considered methionine oxidation as physiological relevant (Munoz, Fernandez-Irigoyen et al. 2008). Interestingly, the tryptic peptide we have analyzed was reported to have a modification on Trp^{61} on that same study (Murray, Taylor et al. 2003). However, we did not observe any modification on this particular residue. We have used mice skeletal muscle mitochondria, while Murray et al. (2003) have used bovine and human heart mitochondria. Thus we may suggest that, at least in mice skeletal muscle mitochondria, the Trp residue is somehow protected against oxidative modification. The deamidation produces a mass shift of less than 1 Da and is not easily detected. Thus, this double observation, further confirms the modification seen in Asn^{69}. Although the modification ratios differ whether we are looking at the single modified or the double modified peptide, the deamidation of Asn69 is more prominent in the NS mouse. Another modified residue in this subunit is Trp^{75}. This residue is markedly prone for oxidation to HTRP in the Y mouse. Oxidation to NFK was also detected and, like the oxidation to HTRP, it is more marked in the Y mouse, decreasing for the NS mouse and decreasing even more in the S mouse (Figure III.7).

Subunit PDSW presented four different residues modified. As stated above, methionine oxidation may be considered an artefact attributed to the sample handling (Froelich and Reid 2008). Since all the samples were subject to the same handling conditions, it is worth noting that in this case the ratios of oxidized methionine/unmodified peptide are so different that the handling-induced artifacts do not explain the pronounced differences found among groups. Thus, one must consider the oxidation of Met^{102} must be physiological relevant in some way, otherwise it would not be so markedly present in the Y mouse. Other modified residues in this subunit are Trp^{4} and Trp^{42}. Trp^{42} is markedly modified to NFK in the non-sedentary aged individuals, supplanting the unmodified peptide. The modification to HTRP was also detected on the three groups, although at lower levels, compared to the oxidation of Trp to NFK. The oxidation of Trp^{4} to HTRP shows a decrease in intensity from the young to old individuals. The presence of the same peptide with Trp^{4} modified to NFK was also found, and follows the same pattern

described for the modification to HTRP. Interestingly, Pro^{1} was also detected simultaneously on the same peptide as being modified to pyroglutamic acid. This is a common N-terminal post-translational modification, enzymatically catalyzed, when Glu or Gln are the first amino acids in the protein sequence (Twardzik and Peterkofsky 1972; Chelius, Jing et al. 2006). However, it may also be the product of Pro oxidation (Uchida, Kato et al. 1990; Berlett and Stadtman 1997). The presence of this modification at the same time than the oxidation of Trp^{4} to HTRP, further confirms the existence of the latter (Figure III.8).

Subunit 30 kDa presented three distinct aminoacids modified. Pro^{222} was found oxidized to pyroglutamic acid and oxidized to glutamic semialdehyde, although with extremely low relative abundances. Prolyl residues were described by Requena et al (2003) as being oxidized to hydroxyproline and consecutively to glutamic semialdehyde, being the latter one of the most common contributors for the carbonyl content of proteins (Requena, Levine et al. 2003). As stated above, Pro can also be oxidized to pyroglutamic acid (Uchida, Kato et al. 1990; Berlett and Stadtman 1997). Given that the pyroglutamic acid is the cyclization dehydration product of glutamic semialdehyde (Smith and Greenberg 1956), Pro may be oxidized to glutamic semialdehyde which, in turn, will be converted into pyroglutamic acid by dehydration. This may explain why glutamic semialdehyde appears to be present in bigger amounts on the Y mouse and not appearing at all in the S mouse, as a more oxidizing environment favours the cyclized form. Another modified amino acid in this subunit is Trp^{238}. These modifications were found in two distinct tryptic peptides but the relative intensities of the mass peaks were almost the same. Once again, this Trp residue was found oxidized to HTRP and NFK. Similarly to Trp^{45} in the MLRQ subunit and Trp^{42} in the PDSW subunit, the NFK modification was more pronounced than the HTRP. The Y mouse also showed a relative amount of modified peptides bigger than its counterparts. The other modified residue in this subunit is Asp^{189}. This amino acid was found modified in residual amounts; nonetheless the Y mouse presented a relatively higher amount of this modified residue (Figure III.9).

The last subunit that was found to have modifications was the 42kDa subunit. Two Trp residues were found modified in this subunit. Trp^{134} was found oxidized to HTRP and NFK. The oxidation of this residue was already reported by Muñoz et al (2008). The relative intensity of these modifications increased from Y to NS and S mice and oxidation to NFK was more frequent than HTRP. Similarly, the oxidation of Trp^{289} followed the same pattern and the oxidation to NFK was more intense than the oxidation to HTRP (Figure III.10).

Unexpectedly, the often reported increase in the oxidative levels associated with aging, and already assessed in Chapter II, was not reflected on the experimental data here presented. Apart from the 42 kDa and B17.2 subunits, the intensity of modified peptides decrease in aged mice relatively to the young. These apparently contradictory results may be explained by the increase in the relative amount of every subunit in S mice, somehow diluting the presence of modified peptides. Indeed, it has been reported that the detectable amount of modified peptides must be over 1% of the total (Nielsen, Savitski et al. 2006; Munoz, Fernandez-Irigoyen et al. 2008). As previously suggested in Chapter II, this further supports the theory that mitochondria may have a mechanism of defence against oxidative stress by increasing the synthesis of the subunits to compensate the lack of functionality due to oxidative damage. Moreover, a closer look at the relative proportions of modified and unmodified peptides shows that subunits PDSW and B17.2 are more affected by oxidative damage than the remaining subunits analyzed (Figure III.2). This further supports our hypothesis that these subunits, which are not involved in the catalytic function of Complex I, may act as anchors essential for the assembly of the complex and are kept in place until they become severely damaged and functionally compromised, while other subunits involved in the catalytic function, are replaced more frequently when damaged. On the other hand, although the software analysis did not showed differences, as the same modifications were found in all groups, when we compare the MS spectra for the same spot on the three groups, visible differences were detected. We observe a positive mass shift in old mice that suggest that we have modified peptides present (Figure III.11). Nonetheless,

the peaks intensity was below the threshold for the software detection and analysis.

In conclusion, several studies have clearly demonstrated that mitochondrial ROS generation increases with old age (Nohl and Hegner 1978; Sohal and Sohal 1991; Kim, McCarter et al. 1996). Complex I of the ETC is a major contributor to reactive oxygen species (ROS) formation in mitochondria (Brandt 2006). Thus, we have identified the 13 locations prone to oxidative damage within Complex I of the ETC and tryptophan was the most susceptible amino acid residue. Additionally, we determined that there is not an evident age- or lifestyle-related pattern for the extent of the modifications, which does not reflect the reported increase in reactive species levels associated with age and physical activity.

CHAPTER IV – General discussion and conclusions

IV. GENERAL DISCUSSION AND CONCLUSIONS

Many diseases, mainly age-related, have been associated with mitochondria, making them a possible target for therapeutics or simply for delay the aging process (Grad, Cepero et al. 2001; Morin, Hauet et al. 2001). Thus, to be able to do this, one must fully understand the age-related mechanisms associated with mitochondria. In this study we aimed to disclose how mitochondria respond to aging and how the practice of moderate exercise throughout life could modify this response. To study the physical activity effect on mitochondria we must choose a tissue that is known to be involved in physical activity and that is rich is mitochondria. Skeletal muscle came as the most abundant and easy to manipulate tissue that fulfils the previous requirements. Other works had already used skeletal muscle mitochondria as a model of study (Cogswell, Stevens et al. 1993; Short, Bigelow et al. 2005; Hood, Irrcher et al. 2006; Menshikova, Ritov et al. 2006; Meany, Xie et al. 2007; Figueiredo, Ferreira et al. 2008); however, none of these studies does such a complete analysis to relate protein oxidative damage to mitochondrial functionality, as the proposed herein.

Our primary strategy was to use 2-DE and mass spectrometry, coupled with other biochemical parameters, namely in-gel activity of respiratory chain complexes, to characterize the differences in the response of mitochondria to aging and physical activity. We analyzed 496 proteins spots resolved by 2-DE, which resulted in 79 proteins identified in 273 spots.

We have observed that aging induces an up-regulation of most respiratory chain proteins, while TCA cycle enzymes are down-regulated in skeletal muscle mitochondrial proteome. On the other hand, lifelong physical activity, resulting in a non-sedentary lifestyle, prevents the observed protein up-regulation for most proteins, but increases the presence of the main antioxidant component – Mn-SOD, thus keeping protein content at levels similar to young individuals with an up-regulated antioxidant system.

Also, we have determined that the non-sedentary lifestyle has a beneficial effect on protein oxidation, reducing the total carbonyl content of

mitochondrial proteins. Despite that, the proteins most affected by age-related carbonylation are Complex III iron-sulphur subunit, Mn-SOD, aspartate aminotransferase, creatine kinase and malate dehydrogenase, while Complex I MLRQ subunit/ATP synthase e chain, cytochrome c and cytochrome c_1 are more affected in the absence of physical activity. It was also observed a relation between the carbonylation and proteins susceptibility to fragmentation, as aconitate hydratase was found present in 34 spots and was one of the most carbonylated proteins.

Regarding mitochondrial functionality, we have showed that it is generally diminished by aging, based on the in-gel activity of Complexes IV and V; however, the non-sedentary lifestyle reverts this tendency. Moreover, the results suggest that the up-regulation of proteins from the respiratory chain may be a mechanism to overcome the age-associated loss of functionality, although it has only been successful for Complex I.

Our second methodological approach allowed us to locate, in Complex I of the respiratory chain, 13 amino acid residues that are susceptible to oxidative modification, distributed through 7 subunits. Interestingly, membrane subunits were found more intensely modified than peripheral subunits and unexpectedly no association was found between number of damaged residues and age vs. lifestyle. Also, tryptophan was the amino acid residue most susceptible to oxidative damage.

As a final conclusion, we have clearly shown that the kind of lifestyle adopted throughout life has severe implications on the mitochondrial proteome of skeletal muscle and on its functionality. Thus, we provide supporting evidence, at a molecular level, for the often described improvements in general health and longevity associated with a lifestyle including regular and moderate physical activity.

V - References

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VI - APPENDIX

VI. APPENDIX

1. Chemicals

Designation	Supplier	Catalog No.
α-cyano-4-hydroxycinnamic acid	Fluka	70990
2,4-Dinitrophenylhydrazine	Sigma	D-2630
3-[N,N-dimethyl(3myristoylaminopropyl)-ammonio]propanesulphate (ASB-14)	Fluka	53327
6-aminohexanoic acid	Sigma	A-7824
Acetic acid (Glacial) 100%, anhydrous P.A.	Merk	1.00063.2511
Acetonitrile (HPLC-gradient grade)	Panreac	221881
Acrylamide 40% solution	Biorad	161-0140
Ammonium hydrogenocarbonate	Riel-de Haën	11213
Ammonium persulphate	Plus One Amersham Biosciences	17-1311-01
ATP	Sigma	A26209
Bis-acrylamide 2% solution	Biorad	161-0142
CHAPS	Plus One Amersham Biosciences	17-1314-01
Coomassie Brilliant Blue G/R250	Fluka	27815 (G250) 27816 (R250)
Cytochrome c	Sigma	C-7752
Diaminobenzidine	Sigma	D-8001
Digitonin	Sigma	D-5628
DL-dithiotreitol	Fluka	43817
Drystrip Cover Fluid	Plus One Amersham Biosciences	17-1335-0
ECL™ Advance Western Blotting Detection	GE Healthcare	RPN2109
Formaldehyde solution, min. 36,5%	Riedel-de Haën	33220
Formic acid, P.A. for mass spectrometry	Fluka	94318
Glycerol 87%	Plus One Amersham Biosciences	17-1325-01
Glycerol, Ultrapure MB grade	USB	16374

Glycine, Ultrapure MB grade	USB	16407
Imidazole	Sigma	I-0125
Immobiline™ DryStrip	GE Healthcare	
Methanol (P.A.)	Fluka	65543
MOLICO Dry non-fat milk	Nestlè	--
NADH	Sigma	N-6785
Nitrotetrazolium Blue chloride (NBT)	Sigma	N-6876
Polyoxyethylene-sorbitan monolaurate (Tween 20)	Sigma	P-5927
Sodium Acetate anhydrous	Fluka	71180
Sodium Chloride (P.A.)	Panreac	131659
Sodium Dodecyl Sulphate	Fluka	71729
TEMED	Plus One Amersham Biosciences	17-1312-01
Thiourea	Riel-de Haën	33717
Tricine	Sigma	T-5816
Trifluoracetic acid	Fluka	91699
Tris	USB	75825
Triton X-100, for electrophoresis	Sigma	T-8532
Trypsin, Sequencing Grade Modified	Promega	V5111
Urea	Plus One Amersham Biosciences	17-1319-01

2. Equipment

Description	**Supplier**
Ettan IPGphor	Amersham Biosciences
miniVE Vertical Electrophoresis System	Amersham Biosciences
SE 600 Dual Cooled Vertical Gel Unit	Amersham Biosciences
ABI 4800 Maldi TOF/TOF Analyzer	Applied Biosystems

3. Reagents and solutions

3.1. 2D-Electrophoresis

Rehydration buffer for isoelectric focusing (10 ml)

Urea ..... 4.8 g (8M)

Thiourea ..... 1.52 g (2M)

CHAPS ..... 200 mg (2%)

ASB-14 ..... 400 mg (4%)

Dissolve in deionised H_2O and adjust the volume to 10 ml.

Prior to use add:

DTT ..... 20 mg/ml (129mM)

IPG buffer ..... 3 µL/ml (0.3%)

Bromophenol Blue (just to add some color to the solution)

Equilibration buffer (200 ml)

1.5M ..... Tris (pH 8.8) 6.7 mL

Urea ..... 72.1 g

Glycerol 87% ..... 69 mL

SDS ..... 4 g

Dissolve in deionised H_2O and adjust the volume to 200 mL

10% APS (ammonium persulfate) (1 ml)

In 1 ml of deionised H_2O dissolve 100mg of APS. Note: prepare fresh before use.

10% SDS (sodium dodecilsulfate) (10 ml)

In 10 ml of deionised H_2O dissolve 1g of SDS.

1.5M Tris (pH 8.8) solution (250 ml)

Tris ..... 45.4 g (1.5M)

Dissolve in deionised H_2O, adjust pH to 8.8 and adjust final volume to 250 ml.

Running buffer (10x) (1 L)

Tris ..... 30.3 g (250 mM)

Glycine ..... 144.2 g (2.5 M)

SDS ..... 10 g (1%)

Dissolve in deionised H_2O and adjust the volume to 1 litre.

Agarose sealing solution (100 ml)

Agarose ..... 0.5 g

Running buffer (10x) ..... 10 ml

Boil in 90 ml of deionised H_2O until complete dissolution, add the running buffer and traces of bromophenol blue and adjust the volume to 100 mL.

15% Acrylamide gels for SDS-PAGE (100 ml - 4 large gels)

Deionised H2O ..... 16.9 ml

Tris-HCl 1,5M pH8,8 ..... 25.0 ml

Acrylamide 40%..... 36.46 ml

Bis-acrylamide 2% ..... 20.10 ml

SDS 10% ..... 1.0 ml

APS 10% ..... 0.5 ml

TEMED..... 0.05 ml

Mix gently and pour quickly between the glass plates, cover with deionised H_2O and wait until polymerization is complete. Note: Acrylamide is a neurotoxin! Always wear gloves.

3.2. BN-PAGE

Colourless cathodal buffer (1 L)

Tricine ..... 9.0 g (50 mM)

Imidazole ..... 0.51 g (7.5 mM)

Dissolve in deionised H_2O and adjust the volume to 1 L with deionised H_2O.

Coloured cathodal buffer (1 L)

Coomassie Blue G250 ..... 0.2 g

Dissolve in 1 L of colourless cathodal buffer

Anodal buffer (5x) (1 L)

Imidazole ..... 1.7 g (25 mM)

Dissolve in deionised H_2O and adjust the volume to 1 L with deionised H_2O.

Gel buffer (3x) (100 ml)

Imidazole ..... 0.5 g (25 mM)

6-aminohexanoic acid ..... 19.7 g (1.5 M)

Dissolve in deionised H_2O and adjust to pH 7.0 and complete the volume to 100 ml with deionised H_2O.

Sample loading buffer (1 ml)

Coomassie Blue G250 ..... 50 mg (5%)

6-aminohexanoic acid ..... 98 mg (750 mM)

Dissolve in deionised H_2O and complete the volume to 1 ml with deionised H_2O, divide in single use aliquots of 100 µl.

3.3. Immunoblotting

Electrotransfer buffer (1x) (1 L)

Tris ..... 3.03 g (25 mM)

Glycine ..... 14.41 g (192 mM)

Dissolve in deionised H_2O and adjust the volume to 800 ml with deionised H_2O. Just prior to use add 200 ml of methanol (20%).

TBS (Tris Buffered Saline) (10x) (1 L)

Tris ..... 12.1 g (100 mM)

NaCl ..... 87.7 g (1.5 M)

Dissolve in deionised H_2O, adjust the pH to 8.0 with HCl and adjust the volume to 1 litre.

TBS-T (Tris buffered Saline – Tween 20) (1x) (100 mL)

TBS (1x) ..... ~100 ml

Tween-20 ..... 50 µl (0.05%)

Blocking solution (100 ml)

TBS-T solution (1x) ..... <100 ml

5% non-fat milk (dry powder) ..... 5 g

Dissolve in TBS-T solution and adjust volume to 100 ml.

Antibody solution (25 ml)

TBS-T solution (1x) ..... 2.5 ml

3% non-fat milk (dry powder) ..... 0.75 g

Dissolve in TBS-T solution and adjust volume to 25 ml. Add antibody, mix gently without vortex, and store at -20 ºC.

3.4. Histochemical staining for in-gel activity

Complex I (10x) (25 ml)

Tris ..... 6.0 mg (2 mM)

NADH ..... 2.5 mg (0.1 mg/ml)

NBT..... 62.5 mg (2.5 mg/ml)

Dissolve Tris in deionised H_2O and adjust to pH 7.4, dissolve the remaining ingredients and complete the volume to 25 ml with deionised H_2O.

Complex IV (10 ml)

DAB ..... 5 mg

Cytochrome c ..... 10 mg

Dissolve in phosphate buffer (pH 7.4) and complete the volume to 10 ml.

Complex V (25 ml)

Tris ..... 0.11 g (35 mM)

Glycine ..... 0.51 g (270 mM)

$MgSO_4$ ..... 42 mg (14 mM)

$Pb(NO_3)_2$ ..... 50 mg (0.2%)

ATP ..... 0.11 g (8 mM)

Dissolve in deionized H_2O, adjust to pH 7.8, complete to 25 ml and adjust to pH 7.4, divide into 3 ml single use aliquots.

3.5. Coomassie staining

Fixing solution (1 L)

Methanol ..... 400 ml (40%)

Acetic acid ..... 100 ml (10%)

Mix with 500 ml of deionised H_2O.

Coomassie colloidal staining solution (1 L)

Coomassie brilliant blue G250 ..... 120 mg (0,12%)

Methanol..... 200 ml

Dissolve in deionised H_2O, add methanol and adjust the volume to 1 litre.

Destaining solution (1 L)

Methanol ..... 250 ml (25%)

Dilute in 750 ml of deionised H_2O.

3.6. Maldi matrix

α-cyan matrix (1 ml)

α-cyano-4-hydroxycinnamic acid ... 5 mg

Acetonitrile..... 500 µl

Trifluoracetic acid ..... 3 µl

H_2O ultrapure ..... 500 µl

Apply 0,5 µl of matrix solution on top equal volume of sample in a maldi plate and let it dry.

4. Procedures

4.1. 2D-PAGE

Isoelectric focusing on IPG strips for first dimension

A given amount of sample is solubilized on rehydration buffer. Then is applied on a ceramic strip holder and the selected IPG strip is applied over the sample. A few drops of Drystrip Cover Fluid are applied over the strip to prevent dissecation and the strip holder is covered and placed on an isoelectric focusing unit. A specific program is selected depending on the size of the strip:

13 cm IPG strips

12h00 rehydration, 20ºC

1h30	gradient – 150V	20ºC, 50µA/strip
1h00	gradient – 500V	
1h00	gradient – 1000V	
3h00	step-n-hold – 8000V	

7 cm IPG strips

12h00 rehydration, 20ºC

1h00	gradient – 150V	20ºC, 50µA/strip
1h00	gradient – 500V	
1h00	gradient – 1000V	
1h30	step-n-hold – 5000V	

SDS-PAGE for second dimension

After isoelectric focusing, the IPG strips are equilibrated to prepare them for the separation on a denaturing environment. The strips are removed from the strip holder and incubated for 15-30 min in equilibration buffer. Then the strips are quickly washed with running buffer, to remove the excess of equilibration buffer, and placed on top of a 15% acrylamide gel. Afterwards, the gel is sealed with an agarose solution and placed on the electrophoresis system with running buffer on both the upper and lower tank. An electric current of 200V is applied and left to run until the tracking

die reaches the bottom of the gel. The gels are then ready for the staining procedures.

4.2. Staining with colloidal coomassie

All the staining steps are preformed with mild agitation. After the electrophoretic run, the gels are fixated with fixing solution for 1 hour and then the solution is replaced with coomassie colloidal staining solution. The gels are left in the staining solution overnight. Followed the staining incubation, the gels are quickly washed with water to remove the excess of staining solution and washed 3-4 times for 30 minutes with destaining solution until the background color is removed.

4.3. In-gel tryptic digestion and acid extraction

Protein spots or bands are excised from the gel and may be immediately prepared for digestion or stored at -20ºC. To prepare the gel pieces for tryptic digestion, the gel pieces are incubated with 50 µl of a 25 mM solution of ammonium hydrogenocarbonate for 30 minutes, then is added an equal volume of acetonitrile and incubated for 30 minutes more. The solution is discarded and these steps are repeated. Finally is added 50 µl of acetonitrile and incubated for 5 minutes until the gel pieces turn white. The acetonitrile is discarded and the gels pieces are dried on a SpeedVac. Then 20µl of a 50 µg/ml solution of trypsin in 25 mM ammonium hydrogenocarbonate are added and the samples are incubated at 37ºC. After 1 hour of incubation are added 30 µl of 25 mM ammonium hydrogenocarbonate and left to incubate at 37ºC overnight. The tryptic digests are then subject to acid extraction as follows: 30 µl of 10% formic acid are added to stop the reaction and start the acid extraction. After 30 minutes of incubation, the solution is removed and transferred for a new tube. Then 50 µl of a 1:1 solution of 10% formic acid/acetonitrile are added to the gel pieces, incubated for 30 min and the solution is transferred for the new tube. This step is repeated once more and the tube with the solution containing the tryptic digests is left to dry on a SpeedVac. Then, the tryptic digests are ressuspended on 2% formic acid/50% acetonitrile and are ready for application on a maldi plate.

4.4. BN-PAGE

BN–PAGE was performed based on the method of Schagger and von Jagow (1991) with minor modifications. First, pellet aliquots of mitochondria (400 µg protein) obtained by centrifugation for 10 minutes at 20,000g were solubilized for 10 min on ice in 40 µl of 50 mM Imidazole, 1 mM EDTA (pH 7.0), 50 mM NaCl, 2 mM 6-aminohexanoic acid, and 12 µl digitonin (6.0 g/g of protein). After centrifugation at 20,000g for 20 min at 4 ºC, the supernatant was retained, combined with BN–PAGE loading dye, and separated on a 4 to 13% acrylamide–bisacrylamide BN–PAGE gel using an SE600 Electrophoresis Unit (Hoefer). For separation, coloured cathode buffer was used until the dye front had reached approximately one-third of the way through the gel before exchange with colourless cathode buffer. Native complexes were separated at 200V for 5 h at 4 ºC.

4.5. Histochemical staining to determine the in-gel activity of the respiratory chain complexes

For in-gel activity and histochemical staining assays of mitochondrial complexes, we follow the procedure described by Zerbetto et al. (1997). Complexes I, IV and V were incubated at 37ºC with their respective staining solutions during 4 hours. After staining, gels were fixed for 15 min with 50% methanol/10% acetic acid, except for Complex V, which was only rinsed with water.